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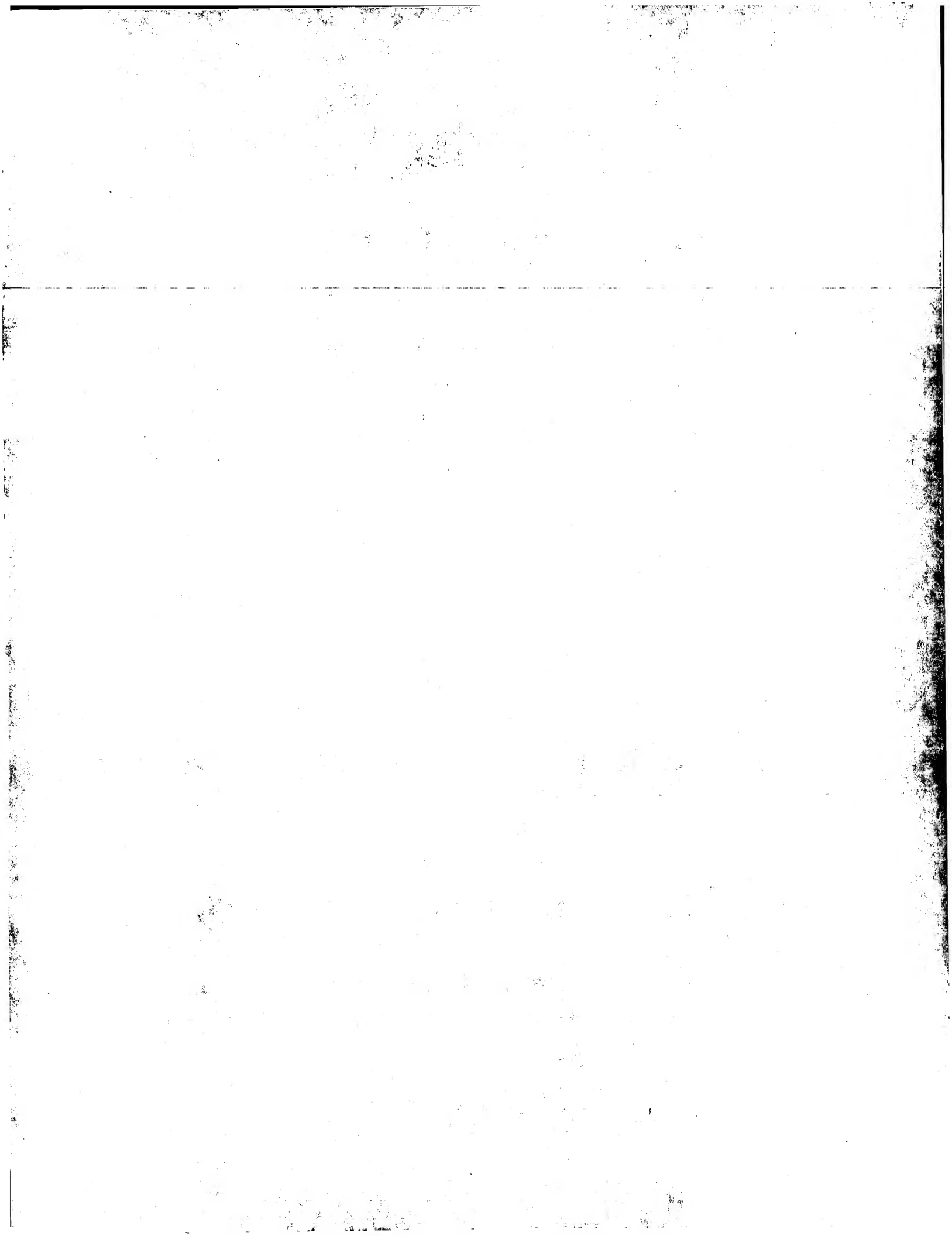
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
17 May 2001 (17.05.2001)

PCT

(10) International Publication Number  
**WO 01/34183 A2**

(51) International Patent Classification<sup>7</sup>: **A61K 38/48**,  
39/395, 31/7088, 31/593, 31/203, 38/18, 31/573, 33/14,  
33/06, C12Q 1/68, A61P 17/00, 27/00, 25/00

(74) Common Representative: **VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW**; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).

(21) International Application Number: **PCT/EP00/11251**

(22) International Filing Date:  
10 November 2000 (10.11.2000)

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:  
99203752.3 10 November 1999 (10.11.1999) EP  
00202776.1 4 August 2000 (04.08.2000) EP

(71) Applicant (*for all designated States except US*):  
**VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW** [BE/BE]; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **DECLERCQ**, Wim [BE/BE]; Pieter Casierstraat 7, B-8510 Marke (BE). **VANDENABEELE**, Peter [BE/BE]; Nijverheidsstraat 18, B-9040 Sint-Amandsberg (BE). **LIPPENS**, Saskia [BE/BE]; Citadellaan 75, B-9000 Gent (BE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *Without international search report and to be republished upon receipt of that report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: **USE OF CASPASE-14 AND CASPASE-14 MODULATORS TO DIAGNOSE AND/OR TREAT SKIN, EYE AND BRAIN DISORDERS**

(57) Abstract: The present invention is based on the finding that caspase-14 is expressed in epithelial tissue such as the epidermis, the epithelial layer of the choroid plexus and the pigmented retinal layer. The invention relates to the involvement of caspase-14 in keratinocyte differentiation and in a properly functioning blood-brain barrier and blood-eye barrier. The present invention specifically relates to nucleic acids encoding caspase-14, caspase-14 protein and modulators of caspase-14 expression, activation and bioactivity that can be used to diagnose and/or treat keratinocyte differentiation disorders, disturbances in the blood-eye barrier and brain disorders.

WO 01/34183 A2

**Use of caspase-14 and caspase-14 modulators to diagnose and/or treat skin, eye  
and brain disorders**

Field of the invention

5 The present invention is based on the finding that caspase 14 is expressed in epithelial tissue such as the epidermis, the epithelial layer of the choroid plexus and the pigmented retinal layer. The invention relates to the involvement of caspase14 in keratinocyte differentiation and in a properly functioning blood-brain barrier and blood-eye barrier. The present invention specifically relates to nucleic acids encoding  
10 caspase-14, caspase-14 protein and modulators of caspase-14 expression, activation and bioactivity that can be used to diagnose and/or treat disorders of keratinocyte differentiation, disturbances in the blood-eye barrier and certain brain disorders.

Background of the invention

15 The epidermis is a stratified squamous epithelium in which keratinocytes are organised in distinct cell layers. The basal cells proliferate, but suprabasal keratinocytes withdraw from the cell cycle and progressively undergo a terminal differentiation program towards the surface of the skin. This keratinocyte differentiation process has several similarities to apoptosis: bcl-2 is downregulated in suprabasal  
20 layers of the epidermis (Lu *et al.*, 1993; Polakowska *et al.*, 1994), c-myc is upregulated (Gandarillas *et al.*, 1997) and in later stages transglutaminases are activated in order to form the cornified envelope (Rice *et al.*, 1992). In the final stage of differentiation during the formation of the cornified layer, the nucleus is condensed and eventually destroyed (McCall and Cohen, 1991; Haake and Polakowska, 1993).

25 Caspase family members play a central role in apoptosis or programmed cell death (PCD) and it was demonstrated that caspases-1, -2, -3, -4 and -7 were expressed in cultured keratinocytes (Takahashi *et al.*, 1998). Weil and colleagues suggested that at least caspase-3 is involved in keratinocyte differentiation and the denucleation process (Weil *et al.*, 1999). Albeit, the terminal differentiation of  
30 keratinocytes does not seem to be a classical apoptosis since these cells don't have any membrane blebbing or DNA fragmentation and are not phagocytosed (Polakowska *et al.*, 1994; Gandarillas *et al.*, 1999). Although these differences indicate that terminal differentiation is not the classical apoptosis it is still possible that both processes use a similar signal transduction pathway. In addition, recent evidence

indicated that caspases also act in differentiation processes that are associated with organelle loss such as lens fiber differentiation and oocyte maturation (Ishizaki *et al.*, 1998; McCall *et al.*, 1998).

5 Mouse and human caspase-14 (Van de Craen *et al.*, 1998) have been described. These caspases have a very restricted expression pattern, this in contrast with all other caspases identified (Van de Craen *et al.*, 1997 and 1998a and b; Humke *et al.*, 1998).

Skin keratinocytes play a central role in the vitamin D endocrine system. Upon exposure to sunlight, the UVB component converts provitamin D<sub>3</sub> to previtamin D<sub>3</sub> that  
10 becomes subsequently thermo-isomerised to vitamin D<sub>3</sub> (Hollick, 1997). Eventually, hydroxylation leads to the formation of the hormonally active form of vitamin D<sub>3</sub>: 1,25(OH)<sub>2</sub> D<sub>3</sub> (Fu *et al.*, 1997). Keratinocytes respond to 1,25(OH)<sub>2</sub> D<sub>3</sub> with growth arrest and differentiation (Hosomi *et al.*, 1983; Bikle and Pillai, 1993; Chen *et al.*, 1995). This observation has led to the successful use of vitamin D analogues in the  
15 treatment of hyperproliferative skin diseases such as psoriasis (van de Kerkhof, 1995; Fogh *et al.*, 1997). Psoriasis, a common skin disorder with a frequency up to 2 % in many populations, is characterised by keratinocyte hyperproliferation with parakeratotic differentiation (Ragaz and Ackerman, 1979; Bata-Csorgo *et al.*, 1993). This epidermal hyperproliferation has not yet been explained on a molecular basis.

20 The brain functions within a chemically well-controlled environment, therefore the brain does not allow most water soluble products of the blood to enter. Both, the choroid plexus and the arachnoid membrane act as barriers between the blood and the cerebrospinal fluid (CSF). The choroid plexus, besides its diffusion restrictive action, actively regulates the concentration of a wide variety of molecules present in  
25 the CSF. The brain ventricles, containing CSF, are covered with a single layer of cells called ependymal cells. This cell layer constitutes the loosely barrier between the CSF and the interstitial fluid and at the choroid plexus passes into the choroid plexus epithelial cells covering capillaries and small blood vessels. Because of the tight junctions between the epithelial cells water-soluble molecules cannot diffuse freely  
30 between the blood and the CSF.

Two eye-blood or blood-ocular barriers regulate exchanges between the blood and the eye: the blood-aqueous barrier residing in the ciliary body and iris, and, the blood-retina barrier. The latter barrier exists at two levels: 1) at the retinal capillaries that supply the inner layers of the retina and that are non-fenestrated and do not allow

the passage of macromolecules, and 2) at the freely permeable choriocapillaries supplying the outer layers of the retina. Breakdown of the blood-ocular barriers is common during intra-ocular disease. In the anterior chamber this leads to an increase in the protein content of the aqueous, which is observed clinically as 'flare' under the slit lamp. Breakdown of the blood-retina barrier at the level of the retinal capillaries and resulting in retinal oedema may occur for many reasons, of which the most important are inflammatory conditions such as uveitis, venous occlusions and diabetic retinopathy, and surgical trauma. Breakdown of the blood-retina barrier at the level of Bruch's membrane and the pigmented epithelium results in accumulation of fluid between the pigmented epithelium and the neuroretina, which thus becomes detached. This occasionally occurs spontaneously in the macular region, but is more often secondary to inflammation or tumours involving the choroids.

#### Summary of the invention.

The current invention is based on the finding that caspase-14 expression is restricted to the suprabasal layers of the skin, the epithelial cells of the choroid plexus and the pigmented retinal layer in the eye. The pigmented retinal layer is situated next to the vascular plexus of the choroid coat of the eye. Hence, as in the choroid plexus in the brain, it is of extreme importance that the exchange of water-soluble molecules between the eye and the blood is tightly controlled. Protection of the eye from invading pathogens or inflammatory factors is an absolute requirement for the preservation of vision (for review Streilein, 1999). Immune privilege of the eye is achieved by special micro-anatomic features (blood-eye barrier), soluble factors secreted by ocular cells and by regulatory molecules constitutively expressed on the surfaces of ocular cells, such as Fas (Griffith *et al.*, 1996). Since caspase-14 is expressed in tissues where cells need to be tightly linked to one another, a functional requirement for caspase-14 expression is needed in the correct formation or functioning of these well controlled barriers, such as the skin, the brain choroid plexus and the retinal layer in the eye. In addition, caspase-14 expression is drastically reduced in the skin plaques of psoriatic patients showing that caspase-14 is involved in the keratinocyte terminal differentiation program leading to normal skin development. Caspase-14 is also expressed in the developing hair follicle, so having a role in hair growth or formation as well, and in the sebaceous gland implicating a role in this gland functioning as well. In view of the above, the present invention thus relates to the modulation of caspase-14 in order to

treat or diagnose keratinocyte differentiation disorders such as cornification disorders, disturbances in the blood-eye barrier and brain disorders. More specifically, the present invention discloses the usage of nucleic acids encoding caspase-14, caspase-14 protein or fragments thereof or modulators of caspase-14 expression, activation and bioactivity such as anti-caspase-14 antibodies, ribozymes, vitamine D, retinoic acid, transforming growth factor-beta (TGF-beta), glucocorticoids, lithium salts and/or calcium salts to treat or diagnose the latter diseases.

#### Brief description of figures and table

**Fig. 1A** shows that over-expression of caspase-14 does not induce apoptosis. GFP and human caspase-14 expressing plasmids were transiently transfected in MCF-7 or HEK293T cells. As a control for apoptotic cell death, cells were transfected with a caspase-8 expressing plasmid. The transfected, GFP positive population was analysed for the presence of apoptotic cells by means of light (L) and fluorescence (F) microscopy. Caspase-8 expressing cells clearly show apoptotic morphology such as nuclear condensation. In contrast, caspase-14 expressing cells show a completely unaffected morphology.

**Fig. 1B and C** demonstrate that caspase-14 is not processed when over-expressed in HEK293T or MCF-7 cells.

B) HEK293T cells or MCF-7 cells were transfected with different plasmids expressing murine or human caspase-14 (see also Fig. 1A). After 24h, lysates were prepared and these were subjected to SDS-PAGE, electroblotted and revealed with a polyclonal anti-caspase-14 antibody.

C) Caspase-3, -6 or -7 were overexpressed in HEK293T cells. Lysates were prepared after 24h and used for immunoblotting with the corresponding anti-caspase antibodies (filled arrow = procaspase; open arrow = large and small subunits of processed caspase).

**Fig. 2** demonstrates the expression of caspase-14 in the mouse embryo.

Immunohistochemistry, with an anti-caspase-14 antibody was performed on transversal sections of 15,5 dpc Balb/c mice. A) expression in the skin, B) expression in the eye, C) expression in the brain (D = dorsal side; dk = differentiated keratinocytes; ec = epithelial layer of the choroid plexus; ep = epidermis; h1 = premature hairbulb; h2 = maturing hairbulb; l = lens; n = nucleus; nl = neural layer of the retina; p = periderm; pl = pigmental layer of the retina; sb = stratum basale; V =

ventral side; vc = vascular plexus of the eye choroid).

**Fig. 3** shows expression of caspase-14 in normal skin and psoriatic plaques.

Normal human skin and skin from psoriasis patients were fixed, embedded in paraffin and sliced. The sections were used for immunohistochemistry with an anti-caspase-14 antibody. A) Normal skin. B) Skin from a psoriasis patient (ep = epidermis; sb = stratum basale; sc = stratum corneum; sg = stratum granulosum; ss = stratum spinosum; n = nucleus; pp = psoriatic plaque). Microscopical magnification is indicated in figures.

**Fig. 4** shows caspase expression pattern in normal mouse skin.

100 µg of protein extracted from skin of Balb/c and C57/B16 mice was used for immunoblotting to analyse the activation of the indicated caspases (filled arrow: procaspase; open arrow: cleaved caspase fragments representing the large p20 subunit).

**Fig. 5** demonstrates that caspase-14 is expressed in differentiated keratinocytes.

Lysates of undifferentiated and differentiated HaCaT cells were used for immunoblotting. A) Differentiation was induced by growing cells postconfluent and the lysates were used for immunoblotting with the indicated antibodies. B) Normal Human Keratinocytes were grown in suspension. Lysates were made at the indicated antibodies and used for immunodetection of caspase-14. As a control for caspase-3 processing lysates were loaded derived from U937 cells treated with TNF and cycloheximide. (filled arrow: procaspase; open arrow: processed caspase).

**Fig. 6** shows the vitamin D dependent up-regulation of caspase-14 expression.

A) HaCaT cells were treated with no,  $10^{-10}$ M,  $10^{-9}$ M,  $10^{-8}$ M,  $10^{-7}$ M or  $10^{-6}$ M vitamin D and lysed after 72h. The lysates were used for immunoblotting with antibodies as indicated. B) Differentiation of HaCaT cells was induced through addition of  $10^{-7}$ M vitamin D. Lysates were made at the indicated time-points and used for immunoblotting with the indicated antibodies. As a control for caspase-3 processing lysates were loaded derived from U937 cells treated with TNF and cycloheximide. (filled arrow: procaspase; open arrow: processed caspase).

**Fig. 7** shows tissue of normal human plexus choroideus, plexus choroideus papilloma and plexus choroideus carcinoma that was fixed, paraffin embedded and sliced. Caspase-14 protein expression was detected by means of immunohistochemistry using a polyclonal anti-caspase-14 antibody. Caspase-14 is present in the epithelial cells of normal plexus choroideus, but is absent or very weakly expressed in plexus

choroideus papilloma and plexus choroideus carcinoma. The top panel of 2 pictures show a normal plexus choroideus, the middle panel of 2 pictures a plexus choroideus papilloma and the lower panel of 2 pictures a plexus choroideus carcinoma. The pictures on the left hand side are controls; the pictures to the right hand side show caspase-14 protein expression.

**Fig. 8** shows the induction of procaspase-14 expression in HaCaT cells by vitamine D, LiCl and hydrocortisone. Hacat cells were grown subconfluent (top figure A) or induced to differentiate by means of post-confluent growth (bottom figure B). The cells were treated with the indicated agents for 72 h. Vitamin D<sub>3</sub> was added to the cells 3 h before retinoic acid treatment. Lysates were prepared and used for western analysis with a polyclonal anti-caspase-14 antibody. Human skin lysate was used as a control for caspase-14 expression.

**Table 1** shows the effect of vitamin D treatment on caspase-14 expression in human psoriatic plaques in stratum basale, stratum spinosum, stratum granulosum and stratum corneum. 1 = control skin; 2 = psoriatic skin before vit D treatment; 3 = psoriatic skin after vit D treatment; - = no staining; +/- = weak or sporadic staining; + = staining; ++ = intens staining; ND = not determined; A, B, C, D, E, F, G are patient numbers (7 patients in total); sample numbers are indicated between brackets.

#### Detailed description of the invention

It is an object of the present invention to modulate the formation or functioning of well controlled barriers such as skin, the brain choroid plexus or retinal layer in the eye based on caspase-14 expression. It is thus an aim of the present invention to use nucleic acids encoding caspase-14, caspase-14 proteins or modulators of caspase-14 bioactivity to modulate keratinocyte differentiation and/or the blood-eye barrier and/or the blood-brain barrier, and as such, to treat disorders of skin and in particular keratinocyte differentiation disorders, disturbances in the blood-eye barrier and brain disorders. The term 'modulate' means control or regulation (up- or down regulation), positively or negatively, of the expression, activation or bioactivity of caspase-14 protein or of its encoding nucleotide sequence. The terms 'nucleic acids encoding caspase-14' refer to a polymeric form of nucleotides, either ribonucleotides or deoxyribonucleotides, encoding for caspase-14. Thus, these terms include double- and single-stranded DNA, and RNA. They also include known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring

nucleotides with an analogue. Specifically, the nucleic acid sequences of the invention comprise a coding sequence for caspase-14 as disclosed in PCT/EP99/04939. A "coding sequence" is a nucleotide sequence that is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA. Also fragments of 'nucleic acids encoding caspase-14' are part of the present invention. These fragments can contain 5, 10, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 or more contiguous nucleotides and must be capable of modulating keratinocyte differentiation and/or the blood-eye barrier and/or the blood-brain barrier. The terms 'modulators of caspase-14 bioactivity' refer in principle to any agent or molecule that has an effect on the amount of caspase-14 protein, the stability of caspase-14 or on the bioactivity of caspase-14 protein in any cell. By 'agent or molecule' it is meant peptides, proteins, carbohydrates or any other small organic or an-organic compound. The present invention thus relates to molecules that can be used to neutralize the activity of caspase-14 by interfering with its synthesis (i.e. transcription) and/or its translation. In this respect, it is clear that some modulators may act directly on the above-mentioned regulatory sequences such as the caspase-14 promotor, or on mRNA encoding caspase-14, or on the translated, non-bioactive caspase-14 polypeptide or on the bioactive caspase-14 protein whereas other modulators, such as vitamine D, act indirectly on the latter targets. Some examples of 'modulators of caspase-14' are: antagonists neutralizing caspase-14 bioactivity, (small) molecules binding on the promoter region of caspase-14 so that binding of a transcription factor on said promotor is inhibited, (small) molecules binding to said transcription factor so that again binding to the caspase-14-promoter is inhibited, anti-sense RNA and DNA molecules and ribozymes that function to inhibit transcription or translation of caspase-14, and molecules interfering with the processing of the caspase-14 polypeptide and, as such, the activation of the caspase-14 polypeptide. The anti-sense RNA and DNA molecules and ribozymes as mentioned above function to inhibit the translation of caspase-14 mRNA and/or to affect transcription of caspase-14 DNA into caspase mRNA. For example, anti-sense RNA and DNA molecules that directly block the translation of mRNA bind to said mRNA and as such prevent protein translation. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary

target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of caspase-14 RNA sequences. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites that include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays. Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize anti-sense RNA constitutively or inducible, depending on the promoter used, can be introduced stably into cell lines.

More specific examples of modulators of the present invention are: antibodies binding to caspase-14, vitamin D or homologues/derivates (deltanoids) thereof as described in Keski-Oja J. 1999 (J. Lab. Clin. Med 133(2):95-97) and Jones et al. 1998 (Physiol. Rev. 78(4):1193-1231), retinoic acid or homologues thereof as described in Vahlquist 1999 (Dermatology 199 suppl 1:3-11), transforming growth factor-beta or homologues thereof as described in Akhurst and Balmain 1999 (J. Pathol. 187 (1):82-90), glucocorticoids and homologues thereof, lithium salts and calcium salts as described in Riccardi 1999 (Cell Calcium 26(3-4):77-83). The term 'antibody' or 'antibodies' relates to an antibody characterized as being specifically binding to caspase-14, with said antibody being preferably a monoclonal antibody or an antigen-binding fragment thereof such as a F(ab')<sub>2</sub>, F(ab) or single chain Fv type, or, any type of recombinant antibody derived thereof. The monoclonal antibodies of the invention can for instance be produced by any hybridoma liable to be formed according to classical methods

from splenic cells of an animal, particularly of a mouse or rat immunized against caspase-14, and of cells of a myeloma cell line, and to be selected by the ability of the hybridoma to produce the monoclonal antibodies recognizing caspase-14 which have been initially used for the immunization of the animals. The monoclonal antibodies according to this invention may also be humanized versions of the mouse monoclonal antibodies made by means of recombinant DNA technology, departing from the mouse and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains. Alternatively the monoclonal antibodies according to this invention may be human monoclonal antibodies. Such human monoclonal antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) re-population of severe combined immune deficiency (SCID) mice as described in PCT/EP 99/03605 or by using transgenic non-human animals capable of producing human antibodies as described in US patent 5,545,806. Also fragments derived from these monoclonal antibodies such as Fab, F(ab)<sub>2</sub> and scFv ("single chain variable fragment"), providing they have retained the original binding properties, form part of the present invention. Such fragments are commonly generated by, for instance, enzymatic digestion of the antibodies with papain, pepsin, or other proteases. Furthermore, it is also well known in the art that monoclonal antibodies, or fragments thereof, can be modified for various uses. For example, the antibodies of the present invention can be labelled with an appropriate label of the enzymatic, fluorescent or radioactive type. It should also be clear that the 'antibodies' of the present invention also include polyclonal antisera prepared against caspase-14.

The present invention thus relates to the usage of nucleic acids encoding caspase-14, caspase-14 protein, modulators of caspase-14 expression, modulators of caspase-14 activation and/or bioactivity for the manufacture of a medicament for treating skin disorders, in particular keratinocyte differentiation disorders, disturbances in the blood-eye barrier and brain disorders. It should also be clear that fragments and variants/homologues of said modulators that are capable of modulating keratinocyte differentiation and/or the blood-eye barrier and/or the blood-brain barrier are part of the present invention.

With respect to skin disorders, it should be clear the normal skin consists of two layers: epidermis and dermis, beneath which is the subcutaneous fat. There are also two types of skin, hair-bearing and non-hair-bearing (glabrous), e.g. palms and soles. The current invention discloses that caspase-14 expression and/or activation occurs in

the skin, more specifically in the epidermis, and that modulation of caspase-14 is essential for the treatment of disorders of the skin, in particular of disorders of the epidermis and/or disorders of keratinocyte differentiation, and more in particular cornification disorders. However, it should be clear that caspase-14 may also be expressed in non-skin epithelium such as mouth epithelium. The present invention thus also relates to the modulation of caspase-14 in the latter non-skin epithelia.

'Cornification' is the normal process (orthokeratosis) of skin differentiation and specifically relates to the formation of the stratum corneum. A disorder of cornification thus relates to a defect in the latter process and can be arbitrarily subdivided into parakeratotic and hyperkeratotic disorders. Hereditary conditions characterized by a cornification disorder are: (1) ichthyosis ('fish skin' disease) referring to a complex group of conditions, all characterized by generalized, non-inflammatory flaky and scaly, thickened skin, (2) bullous ichthyiform erythroderma referring to solitary or localized lesions identical to epidermolytic hyperkeratosis present in the skin, (3) Darier disease or follicular keratosis, (4) Hailey-Hailey (benign familial chronic pemphigus), (5) Grover disease (transient acantholytic dermatosis), (6) epidermolysis bullosa (mechano-bullous diseases) and (7) Miliaria (M. crystalline, M. rubra, M. profunda).

In addition there is a group of conditions, called psoriasiform lesions, in which the pathological features are characterized by focal lamellar parakeratosis, regular acanthosis, prolongation of rete ridges and 'squirting papillae'. Psoriasis is an example of such a condition and the present invention discloses that caspase-14 expression in psoriatic skin is drastically down-regulated. So strategies to up-regulate caspase-14 expression may prove to have an important therapeutic contribution to diseases involving parakeratosis such as psoriasis.

Lichenoid lesions characterized by basal cell liquefaction and a 'band-like' chronic inflammatory infiltrate in the papillary dermis and dermatitic (eczematous) lesions are other groups of conditions. Blistering eruptions or pemphigus disorders form another interrelated group of lesions in which blisters involve the epidermis and are characterized by acantholysis, i.e. separation of epithelial cells from each other through loss of intercellular substances and disruption of the desmosomes. This results in individually separated, smooth-surfaced epidermal cells. Two types of pemphigus are recognized: 1) pemphigus foliaceus with its variant, Fogo Selvagem, and 2) pemphigus vulgaris with its variant, pemphigus vegetans.

Since the expression of caspase-14 is also observed in the sebaceous glands, modulation of caspase-14 is useful in treating disease conditions affecting the pilosebaceous follicle such as folliculitis, acne vulgaris, keratosis pilaris, lichen spinulosus, rosacea which is a common condition with a variable clinical appearance including facial flushing, erythema, papules, and pustules, pityriasis rubra pilaris, alopecia including alopecia areata and scarring alopecias. Caspase-14 modulation may also be essential for diagnosing and/or treating tumours of the skin such as epidermal naevi, organoid naevus (naevus sebaceous of Jadassohn), seborrheic keratosis (basal cell papilloma), melanoacanthoma, inverted follicular keratosis, squamous cell papilloma, clear cell acanthoma, epidermal cysts, solar keratosis, bowen disease, squamous cell carcinoma, keratoacanthoma, basal cell carcinoma, basosquamous cancer, lentigo, melanocytic naevus, malignant melanoma, eccrine gland tumours such as eccrine poroma, syringoma, cylindroma, eccrine hidrocystoma, eccrine spiradenoma, hidradenoma and sweat gland carcinoma, tumours of apocrine glands and the pilosebaceous unit such as hidradenoma papilliferum, syringocystadenoma papilliferum, apocrine carcinoma, extra-mammary Paget disease, trichofolliculoma, trichoepithelioma, pilomatixoma, trichilemmoma, sebaceous gland neoplasms and sebaceous hyperplasia. Thus, caspase-14 expression may also be involved in the development of keratinocyte tumors and a protocol can be developed to up-regulate the caspase-14 expression in tumor cells so that denucleation, and a subsequent eradication of said tumor cells, occurs.

Excessive cornification can also take place in the multi-layer epithelium of the mouth the airway and the vagina so that modulation of caspase-14 expression and/or activation also assists in curing these disease processes.

It is clear that modulation of caspase-14 expression and/or activation can also be used in veterinary applications such as treatment of disorders of ectodermal extrusions such as feathers, horns and nails.

Moreover, modulation of caspase-14 expression and/or activation can be used to treat the process of wound healing and to treat patients receiving skin transplants. For example in wound healing a high speed of skin cornification is desired to avoid infection of the skin and therefore an up-regulation of caspase-14 expression and/or activity is beneficial. On the other hand in skin transplant processes it is desirable that the normal skin cornification is delayed before a successful transplantation is carried

out and therefore a down-regulation of caspase-14 and/or activity is more beneficial. Also in vitro skin propagation systems benefit from reduced cornification to allow better growth and expansion of the skin samples.

With respect to disturbances in the blood-eye barrier it is clear from the present invention that caspase-14 expression is specifically observed in the pigmented epithelium of the retina and that under- or over-expression of caspase-14 may result in eye-disease. A non-limited list of such diseases which are due to a malfunctioning of the blood-retina barrier or where a reduction in blood-retina barrier function takes place include (1) glaucoma, (2) diseases, due to various infective agents, such as conjunctivitis, (3) uveitis, (4) Fuchs heterochromic iridocyclitis, (5) sympathetic ophthalmitis, (6) lens-induced endophthalmitis, (7) diseases of the retina such as diabetic eye disease, retinopathy of prematurity, inherited retinal degeneration, retinitis pigmentosa, age-related macular degeneration, senile macular degeneration and disciform degeneration, (8) cataract, (9) intra-ocular tumours such as malignant melanomas, hemangiomas and retinoblastomas (10) multiple sclerosis, (11) ischemia, and (12) AIDS.

With regard to brain disorders, it is clear that the choroids plexus is present within each of the four ventricles of the brain and is responsible for the production of cerebrospinal fluid (CSF). The choroids plexus has a characteristic, lobulated appearance and consists of a single continuous layer of epithelial cells overlying a vascular central core. Choroidal epithelial cells are derived from the ependymal lining of the ventricles. The choroids epithelium consists of a single continuous layer of simple cuboidal cells resting on a basal lamina. The lateral surfaces of adjoining cells are bound by tight junctions obliterating the intercellular space and forming a barrier to extracellular movement of substances to, or from, the CSF. This barrier function that is normally served by brain capillaries is in the choroids plexus shifted to the epithelium itself. The present invention discloses that caspase-14 expression is specifically present (upregulated) in the cytoplasm of the choroidal epithelial cells and that modulation of caspase-14 expression and/or activation is involved in the modulation of the barrier function of the choroids plexus. In man, the normal pressure of CSF as measured in the recumbent position by lumbar puncture varies from 25-70 mm water in infants and from 65-195 mm water (5-15 mm Hg) in adults. Elevations of this CSF pressure may be an indication of a neural pathology like a tumor such as a papilloma or a carcinoma (see examples). An excess in CSF intracranially can result in

hydrocephalus and an excess in CSF pressure can also be the result of head injury, meningitis or idiopathic causes. Untreated, elevated CSF pressure leads to progressive neurological dysfunction, coma, and eventually death. A reduction in blood-brain barrier takes also place in the following diseases: cerebral malaria, traumatic brain injury, acute encephalopathy and encephalitis related to influenza virus infection, multiple sclerosis, acute colitis, AIDS, cerebral ischemie, Alzheimer's disease and acute hypertension. The modulation of caspase-14 expression can thus be used to treat the above-described disorders. Modulation of the choroids epithelial barrier through caspase-14 modulation can also be used for the treatment of neuronal disorders such as Alzheimer's disease or Parkinson's disease where it is necessary that a medicament efficiently crosses the blood-brain barrier or where it is necessary that the crossing of the blood-brain-barrier of a compound or medicament has to be inhibited.

Thus, in certain instances when medication is required, it might be desirable that said barrier is (temporally) less stringent in order to let penetrate medicaments having a therapeutic action into the brain and/or eye respectively.

The present invention also relates to the usage of nucleic acids hybridising with nucleic acids encoding for caspase-14 or fragments thereof, or, molecules, such as antibodies, specifically binding to caspase-14 protein or fragments thereof for the manufacture of a diagnostic assay for skin disorders, in particular keratinocyte differentiation disorders, disorders in the blood-brain barrier and/or disorders in the blood-brain barrier. Thus a nucleic acid, or a fragment thereof, encoding for caspase-14 can be used as a probe for hybridisation to, for example, nucleic acids derived from a skin sample of an animal or a human. It can be also expected that mutations in the caspase-14 gene may lead to skin disorders and that these mutations can be detected by hybridisation with the natural occurring caspase-14 gene or with an oligonucleotide comprising said mutation. Alternatively the diagnostic method can be carried out on the protein level. This can be done by using molecules that specifically bind to caspase-14 protein or a fragment thereof.

Typically such a diagnostic method comprises the steps: (i) measuring the level of caspase-14 in an appropriate skin sample derived from an animal or a human that is to be diagnosed, and then (ii) comparing the level found with the corresponding level (normal level, normal concentration range) for apparently healthy animals or humans (normal individuals). If the level found deviates from the normal level this is an

indication that the animal or human suffers from some abnormal condition of skin disorder. A raised level will be indicative of hyperkeratinization while subnormal levels may be found in connection with parakeratotic syndromes such as psoriasis. The measurement of caspase-14 protein can in principle be performed by any method that provides the satisfactory sensitivity, precision, specificity etc. It is believed that immunoassays are the most preferred methods. Immunoassays comprise bringing the sample suspected of containing an abnormal level of caspase-14 in contact with an antibody specific for caspase-14 (anti-caspase-14 antibody) in an assay medium under conditions permitting formation of an immune complex comprising caspase-14 and the anti-caspase-14 antibody. The complex formed is thereafter determined by per se known methods to give a quantitative or qualitative measure of the caspase-14 level in the assay medium that in turn is a measure of the caspase-14 level in the sample. In these types of assays the complex as such may be measured or it may be measured by aid of a biospecific, affinity reactant labelled with an analytically detectable substance (label), said reactant (and its label) being capable of becoming specifically incorporated into the complex. Examples of suitable biospecific affinity reactants that can be labelled are anti-caspase-14 antibodies, caspase-14 as such, anti-antibodies (anti-anti-caspase-14 antibody) directed against constant regions of an antibody that is present in the complex formed, Proteins A and G etc. Examples of detectable substances (labels) that may be used are luminescers, chromophors, fluorophors, enzymes, enzyme substrates, cofactors, coenzymes, radioactive isotopes, particles (metallic or non-metallic), biotin (detected by its reaction with avidin) etc. Some labels change their signal when becoming incorporated into the immune complex while others do not. The former type of labels provides homogeneous immune assays in which there is no need to separate the label incorporated into the complex from the label not incorporated. The latter type of labels demands the separation to be carried out, for instance by insolubilizing the complex in which the label is or is to be incorporated (heterogeneous assays). In order to achieve an insolubilized complex that contains the label, precipitating agents such as polyethylene glycol and insolubilized and insolubilizable biospecific affinity reactants binding to the complex may be used. Of course this latter type shall not insolubilize the labelled biospecific affinity reactant as such. The artisan in the field is capable of selecting the appropriate immunoassay protocols, for instance homogeneous or heterogeneous variants, order and type of addition and incubation steps etc. The main point is that the

amount of reactants added must be such that the amount of label incorporated into the complex or not incorporated into the complex will reflect the caspase-14 level in the sample. Normal assay conditions are aqueous media with or without non-disturbing water-miscible co-solvents, temperatures within 0-40 °C and pH-values within 4-10.

5 The anti-caspase-14 antibody used may be prepared by standard techniques well-known for other antibodies. The term encompasses polyclonal as well as monoclonal antibodies and antibodies produced by recombinant techniques. The term anti-caspase-14 antibody means an antibody preparation reacting specifically with caspase-14, such as in monomeric forms, caspase-14 as included in di-, multi or  
10 heteromeric forms and/or fragments of monomeric caspase-14 exhibiting caspase-14 unique determinants and epitopes. The present invention discloses that the above-described molecules can be used as a medicament for treatment of diseases as described above or can be used to treat said diseases. In this respect, the present invention also relates to a pharmaceutical composition comprising modulators of  
15 caspase-14 bioactivity, nucleic acids encoding caspase-14 and/or caspase-14 protein. It should be clear that the therapeutic method of the present invention against for example skin disorders can also be used in combination with any other skin therapy known in the art such as PUVA-therapy or retinoic acid treatment. The term 'medicament to treat' relates to a composition comprising molecules as described  
20 above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat diseases as indicated above. The administration of a compound, an antagonist or agonist of the caspase-14 or a pharmaceutically acceptable salt thereof may be by way of topical, oral, inhaled or parenteral administration. The active compound may be administered alone or preferably  
25 formulated as a pharmaceutical composition.

An amount effective to treat the disorders described above depends on the usual factors such as the nature and severity of the disorders being treated and the weight of the mammal. However, a unit dose will normally contain 0.01 to 50 mg for example 0.01 to 10 mg, or 0.05 to 2 mg of Caspase-14 agonist or antagonist or a  
30 pharmaceutically acceptable salt thereof. Unit doses will normally be administered once or more than once a day, for example 2, 3, or 4 times a day, more usually 1 to 3 times a day, such that the total daily dose is normally in the range of 0.0001 to 1 mg/kg; thus a suitable total daily dose for a 70 kg adult is 0.01 to 50 mg, for example 0.01 to 10 mg or more usually 0.05 to 10 mg. It is greatly preferred that the compound

or a pharmaceutically acceptable salt thereof is administered in the form of a unit-dose composition, such as a unit dose topical, oral, parenteral, or inhaled composition. Such compositions are prepared by admixture and are suitably adapted for topical, oral, inhaled or parenteral administration, and as such may be in the form of tablets, capsules, oral liquid preparations, powders, granules, lozenges, reconstitutable powders, injectable and infusable solutions or suspensions or suppositories or aerosols. Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, colourants, flavourings, and wetting agents.

10 The tablets may be coated according to well-known methods in the art.

Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycollate. Suitable lubricants include, for example, magnesium stearate. Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulphate. These solid oral compositions may be prepared by conventional methods of blending, filling, tableting or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavouring or colouring agents.

30 Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating.

Preferably, compositions for inhalation are presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In

such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. A favoured inhaled dose will be in the range of 0.05 to 2 mg, for example 0.05 to 0.5 mg, 0.1 to 1 mg or 0.5 to 2 mg.

- 5 For parenteral administration, fluid unit dose forms are prepared containing a compound of the present invention and a sterile vehicle. The active compound, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing.
- 10 Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to
- 15 ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the active compound. Where appropriate, small amounts of bronchodilators for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and aminophylline and
- 20 corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included. As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

The present invention further provides a pharmaceutical composition for use in the treatment and/or prophylaxis of herein described disorders which comprises a

25 molecule or a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable solvate thereof, and, if required, a pharmaceutically acceptable carrier thereof. Another pharmaceutically acceptable composition is an inhalation composition, suitably in unit dosage form. Yet another pharmaceutical acceptable composition for treatment of skin disorders is a topical composition, suitable in unit

30 dosage form.

Genetic constructs of caspase-14 or functional fragments thereof can also be used in gene therapy. A 'genetic construct' means that the coding information of caspase-14 or a functional fragment thereof is operably linked to elements known in the art that can provide transcription of the caspase-14, such as a promoter and/or enhancer

sequence. Gene therapy means the treatment by the delivery of therapeutic nucleic acids to patient's cells. This is extensively reviewed in Lever and Goodfellow 1995; *Br. Med Bull.*, 51, 1-242; Culver 1995; Ledley, F.D. 1995. *Hum. Gene Ther.* 6, 1129. To achieve gene therapy there must be a method of delivering genes to the patient's cells and additional methods to ensure the effective production of any therapeutic genes. There are two general approaches to achieve gene delivery; these are non-viral delivery and virus-mediated gene delivery. As an example, but not limited to this, is the use of a virus-mediated gene delivery system with replication defective retroviruses to stably introduce genes into patient's cells (see also further). In human skin, ceramides are the major lipid constituents of the stratum corneum. Together with the neutral lipids, they form broad laminated intercellular sheets and play a crucial role in the skin barrier function. Ceramide-containing dermato-cosmetic products have been suggested for use in dermato-cosmetic applications such as a restoration of a disturbed skin barrier function. Also in gene therapy ceramides can be added to compounds activating the activity of caspase-14 or ceramides can be added to vectors comprising nucleic acids encoding caspase-14. The application of ceramides can facilitate the topical administration.

The skin, particularly the epidermis, is appealing as a target tissue for delivery of polynucleotides. For example, skin can be been successfully used as a site for genetic immunization (i.e., immunization by administration of an antigen-encoding sequence) (see, e.g., U.S. Pat. No. 5,589,466; Robinson et al. 1997 *Sem Immunol* 9:217-83; Johnston et al. 1994 *Meth Cell Biol* 43:353-65; Barry et al. 1997 *Vaccine* 15:788-91; Sundaram et al. 1996 *Nucl. Acids Res.* 24:1375-7; Moelling 1997 *Cytokines Cell Mol. Ther.* 3:127-35). Second, skin is an attractive target organ due to its accessibility, thereby providing one of the easiest routes of administration. Moreover, because it is a stratified epithelium, skin allows for the possibility of targeting gene expression to either differentiated or proliferative cells, depending upon the desired effect of gene product expression. In addition, epidermal biology is relatively well-characterized at both the cellular and molecular levels. For example, the regulatory sequences of the keratins have been used to express a variety of exogenous genes in the epidermis of transgenic mice, and are readily adaptable for expression in other organisms (Greenhalgh et al. 1994 *J. Invest. Dermatol.* 103:63S-69S; Vassar et al. 1991 *Genes Dev* 5:714-27; Bailleul et al. 1990 *Cell* 62:697-708).

Further to the present invention it would be desirable to use for example, but not-

limiting, a recombinant adenoviral vector wherein the expression of caspase-14 is under control of a strong, constitutive or inducible promoter which could be used to treat parakeratotic skin diseases such as psoriasis. Alternatively a recombinant viral vector could be constructed where a dominant negative caspase-14 or an antisense construct of caspase-14 or a ribozyme against caspase-14 is under control of a strong promoter. The latter recombinant vector could be used to treat for example skin disorders of hyperkeratinization.

Conventional methods for delivery of polynucleotides for expression in the skin include invasive, semi-invasive, or non-invasive methods. Invasive methods involve breaking the skin or otherwise disrupting or bypassing the epidermal barrier. Conventional invasive methods include needle injection (U.S. Pat. No. 5,589,466; Masayuki et al. 1996 FEMS Immunol Med Microbiol 14:221-30; Ciernik et al. 1996 Hum Gene Ther 7:893-9), particle bombardment ("gene gun;" Vahlsing et al. 1994 J. Immunol Meth 175:11-22; Cheng et al. 1993 Proc. Natl. Acad. Sci. USA 90:4455-9; Sundaram et al. 1996 Nucl. Acids Res. 24:1375-7; Johnston et al. 1994 Meth Cell Biol 43:353-65), and jet injection (Furth et al. 1995 Molec Biotech 4:121-7). Expression of an exogenous DNA has also been accomplished by direct application of DNA or DNA-liposome complexes to incisional wounds (Sun et al. 1997 J Invest Dermatol 108:313-8). Semi-invasive methods involve permeabilization of the epithelium through either mechanical or chemical means. For example, one successful semi-invasive method involves the application of a pulsed electric field to the skin (Zhang et al. 1996 Biochem Biophys Res Commun 220:633-6). The invasive and semi-invasive methods generally deliver the polynucleotide in the form of naked DNA (see, e.g., U.S. Pat. No. 5,589,466). Non-invasive methods include topical application of a DNA-containing formulation that contains transfection-facilitating molecules. Examples of such formulations include liposomes (Li et al. 1995 Nature Med 1:705-6; Alexander et al. 1995 Human Mol Genet 4:2279-85). In general, conventional non-invasive methods involve pretreatment of the skin to remove hair (e.g., by shaving and/or use of a depilatory) (Li et al. 1995 Nature Med 1:705-6; Alexander et al. 1995 Human Mol Genet 4:2279-85).

Although conventional methods hold great promise for delivery of gene products to the skin of local and systemic effects, the more complicated the delivery method or the delivery formulation, the more difficult application of these methods and formulations will be in the field. For example, a genetic construct comprising the caspase-14 polynucleotide sequence preferred for use in the field would be one that requires no

special equipment, such as instruments for breaking the skin to deliver the DNA, and further involves no special formulation that might require special handling. Recent methods for delivery of polynucleotides to skin cells do not require special formulations or invasive procedures to facilitate delivery of the genetic material into skin cells. An  
5 example of such a method is fully described in US 6,087,341.

The topical formulations of caspase-14 (polynucleotide constructs or the purified caspase-14 protein) include those pharmaceutical forms in which the composition is applied externally by direct contact with the skin surface to be treated. A conventional pharmaceutical form for topical application includes a soak, an ointment, a cream, a  
10 lotion, a paste, a gel, a stick, a spray, an aerosol, a bath oil, a solution and the like. Topical therapy is delivered by various vehicles, the choice of vehicle can be important and generally is related to whether an acute or chronic disease is to be treated. As an example, an acute skin proliferation disease generally is treated with aqueous drying preparations, whereas chronic skin proliferation disease is treated with hydrating  
15 preparations. Soaks are the easiest method of drying acute moist eruptions. Lotions (powder in water suspension) and solutions (medications dissolved in a solvent) are ideal for hairy and intertriginous areas. Ointments or water-in-oil emulsions, are the most effective hydrating agents, appropriate for dry scaly eruptions, but are greasy and depending upon the site of the lesion sometimes undesirable. As appropriate,  
20 they can be applied in combination with a bandage, particularly when it is desirable to increase penetration of the caspase-14 composition into a lesion. Creams or oil-in-water emulsions and gels are absorbable and are the most cosmetically acceptable to the patient. (Guzzo et al, in Goodman & Gilman's Pharmacological Basis of Therapeutics, 9.sup.th Ed., p. 1593-15950 (1996)). Cream formulations generally  
25 include components such as petroleum, lanolin, polyethylene glycols, mineral oil, glycerin, isopropyl palmitate, glyceryl stearate, cetearyl alcohol, tocopheryl acetate, isopropyl myristate, lanolin alcohol, simethicone, carbomen, methylchlorisothiazolinone, methylisothiazolinone, cyclomethicone and hydroxypropyl methylcellulose, as well as mixtures thereof.

30 Other formulations for topical application include shampoos, soaps, shake lotions, and the like, particularly those formulated to leave a residue on the underlying skin, such as the scalp (Arndt et al, in Dermatology In General Medicine 2:2838 (1993)).

In general, the concentration of the caspase-14 composition in the topical formulation is in an amount of about 0.5 to 50% by weight of the composition, preferably about 1

to 30%, more preferably about 2-20%, and most preferably about 5-10%. The concentration used can be in the upper portion of the range initially, as treatment continues, the concentration can be lowered or the application of the formulation may be less frequent. Topical applications are often applied twice daily. However, once-  
5 daily application of a larger dose or more frequent applications of a smaller dose may be effective. The stratum corneum may act as a reservoir and allow gradual penetration of a drug into the viable skin layers over a prolonged period of time.

In a topical application, a sufficient amount of caspase-14 must penetrate a patient's skin in order to obtain a desired pharmacological effect. It is generally understood that  
10 the absorption of drug into the skin is a function of the nature of the drug, the behaviour of the vehicle, and the skin. Three major variables account for differences in the rate of absorption or flux of different topical drugs or the same drug in different vehicles; the concentration of drug in the vehicle, the partition coefficient of drug between the stratum corneum and the vehicle and the diffusion coefficient of drug in  
15 the stratum corneum. To be effective for treatment, a drug must cross the stratum corneum which is responsible for the barrier function of the skin. In general, a topical formulation which exerts a high in vitro skin penetration is effective in vivo. Ostrenga et al (J. Pharm. Sci., 60:1175-1179 (1971) demonstrated that in vivo efficacy of topically applied steroids was proportional to the steroid penetration rate into dermatomed  
20 human skin in vitro.

A skin penetration enhancer which is dermatologically acceptable and compatible with caspase-14 can be incorporated into the formulation to increase the penetration of caspase-14 from the skin surface into epidermal keratinocytes. A skin enhancer which increases the absorption of aminoguanidine into the skin reduces the amount of  
25 aminoguanidine needed for an effective treatment and provides for a longer lasting effect of the caspase-14 formulation. Skin penetration enhancers are well known in the art. For example, dimethyl sulfoxide (U.S. Pat. No. 3,711,602); oleic acid, 1,2-butanediol surfactant (Cooper, J. Pharm. Sci., 73:1153-1156 (1984)); a combination of ethanol and oleic acid or oleyl alcohol (EP 267,617), 2-ethyl-1,3-hexanediol (WO  
30 87/03490); decyl methyl sulphoxide and Azone.RTM. (Hadgraft, Eur. J. Drug. Metab. Pharmacokinet, 21:165-173 (1996)); alcohols, sulphoxides, fatty acids, esters, Azone.RTM., pyrrolidones, urea and polyols (Kalbitz et al, Pharmazie, 51:619-637 (1996)); terpenes such as 1,8-cineole, menthone, limonene and nerolidol (Yamane, J. Pharmacy & Pharmacology, 47:978-989 (1995)); Azone.RTM. and Transcutol

(Harrison et al, Pharmaceutical Res. 13:542-546 (1996)); and oleic acid, polyethylene glycol and propylene glycol (Singh et al, Pharmazie, 51:741-744 (1996)) are known to improve skin penetration of an active ingredient.

Levels of penetration of an caspase-14 composition can be determined by techniques known to those of skill in the art. For example, radio-labeling of caspase-14, followed by measurement of the amount of radio-labelled caspase-14 absorbed by the skin enables one of skill in the art to determine levels of the composition absorbed using any of several methods of determining skin penetration of the test compound. Publications relating to skin penetration studies include Reinfenrath, W G and G S Hawkins. The Weanling Yorkshire Pig as an Animal Model for Measuring Percutaneous Penetration. In: Swine in Biomedical Research (M. E. Tumbleson, Ed.) Plenum, New York, 1986, and Hawkins, G. S. Methodology for the Execution of In Vitro Skin Penetration Determinations. In: Methods for Skin Absorption, B W Kemppainen and W G Reifenrath, Eds., CRC Press, Boca Raton, 1990, pp.67-80; and W. G. Reifenrath, Cosmetics & Toiletries, 110:3-9 (1995).

For some applications, it is preferable to administer a long acting form of caspase-14 composition using formulations known in the arts, such as polymers. Caspase-14 can be incorporated into a dermal patch (Junginger, H. E., in Acta Pharmaceutica Nordica 4:117 (1992); Thacharodi et al, in Biomaterials 16:145-148 (1995); Niedner R., in Hautarzt 39:761-766 (1988)) or a bandage according to methods known in the arts, to increase the efficiency of delivery of the drug to the areas to be treated.

Optionally, the topical formulations of this invention can have additional excipients for example; preservatives such as methylparaben, benzyl alcohol, sorbic acid or quaternary ammonium compound; stabilizers such as EDTA, antioxidants such as butylated hydroxytoluene or butylated hydroxanisole, and buffers such as citrate and phosphate.

Other therapeutic agents suitable for use herein are any compatible drugs that are effective for the intended purpose, or drugs that are complementary to the caspase-14 formulation. As an example, the treatment with an formulation of this invention can be combined with other treatments such as a topical treatment with corticosteroids, calcipotrine, coal tar preparations, a systemic treatment with methotrexate, retinoids, cyclosporin A and photochemotherapy. The combined treatment is especially important for treatment of an acute or a severe skin proliferation disease. The caspase-14 formulation utilized in a combination therapy may be administered

simultaneously, or sequentially with other treatment, such that a combined effect is achieved.

Unless stated otherwise in the Examples, all recombinant DNA techniques are performed according to protocols as described in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY or in  
 5 Volumes 1 and 2 of Ausubel et al. (1994), Current Protocols in Molecular Biology, Current Protocols.

The present invention is further described by reference to the following non-limiting examples whereas a material and method section is enclosed for sake of clarity too.

10

## Examples

### Materials and methods

#### ***Human caspase-14 cDNA cloning***

Human skin RNA was prepared from cultured normal human keratinocytes, first strand  
 15 cDNA was prepared using the SuperScript Preamplification System for First strand cDNA synthesis (Gibco BRL). This keratinocyte cDNA was used to obtain a fragment containing full length human procaspase-14 or procaspase-14 $\Delta$ p (procaspase-14 lacking the putative prodomain consisting of 13 amino acids) by means of PCR technology  
 [forward PCR primer:  
 20 ATAAGAATGCGGCCGCAAGCAATCCGCGGTCTTTGGAAGAGGAG; forward PCR  $\Delta$ p primer: ATAAGAATGCGGCCGCAATGTCAGGTGCCCGCCTGGCCCTA; reverse PCR primer: GCTAGCTAGCCTGCAGATACAGCCGTTTCCGGAG; hcaspase-14 matching sequences are indicated in bold. These NotI/NheI digested amplicons were provided with a N-terminal flag tag and a C-terminal myc tag in a modified pCDNA3 vector  
 25 (Invitrogen, San Diego, CA, USA) to generate the pCDNA-flag-hCASPASE-14-myc and pCDNA-flag-hCASPASE-14 $\Delta$ p-myc vectors.

#### ***Transient transfection of HEK293T or MCF-7 and detection of apoptotic cells***

HEK293T is a human embryonal kidney carcinoma stably transfected with the SV40 T-antigen (DuBridge et al., 1987). These cells were cultured in Dulbecco's modified  
 30 Eagle's medium containing, 10% fetal calf serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Human breast carcinoma MCF-7 cells were maintained in RPMI1640 containing 5% fetal calf serum, 5% newborn calf serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin.

5x10<sup>5</sup> HEK293T or MCF-7 cells were seeded per well (6-well plate) and transiently transfected, 1µg of total plasmid DNA, by the lipofection method (FuGene, Boehringer). The cells were co-transfected with pEGFP.N1 (a plasmid constitutively expressing green fluorescent protein (GFP)) and pCDNA3flag (control vector), pSVsportmC14 (Van De Craen *et al.*, 1998a), pCDNA3-mCASP-14 (Van De Craen *et al.*, 1998a), pCDNA3-flag-hCASP-14-myc, pcDNA3-flag-hCASP-14ΔP-myc or pCAGGS-mCASP-8 (Van de Craen *et al.*, 1998b). 24h after transfection the GFP-positive population was analysed for apoptotic morphology by means of fluorescence microscopy.

#### 10 **Immunohistochemistry**

Mouse embryos and skin tissues from humans and C57Bl/6 mice were fixed in 4% para-formaldehyde, paraffine embedded and sliced (4-6µm). Endogenous peroxidase was blocked with Peroxidase Blocking Reagent (Dako). Both murine and human caspase-14 were stained by a rabbit polyclonal antibody originally raised against recombinant, bacterially expressed murine caspase-14. All antibodies were diluted in phosphate buffered saline (PBS) and the primary antibody was detected by an indirect peroxidase antibody conjugate technique. The sections were incubated with a rat anti-rabbit (Dako) for 45 minutes. 3- amino- 9- ethyl carbazole (AEC, Dako) was used as a chromogen for demonstration of the complex. The specificity of the immunohistochemical reactions was checked by omitting the primary antibody or substituting the anti-caspase-14 antibody by an unrelated antibody at the same concentration.

#### **Keratinocyte culture**

HaCaT cells were grown in DMEM supplemented with 10% FCS. Normal human keratinocytes (NHK) were isolated from foreskins of young donors as described (Kitano and Okada, 1983). NHK cells were grown in Keratinocyte Serum Free medium (GIBCO BRL) with a calcium concentration of 0.09 mM and supplemented with 50 µg of bovin pituitary extract per ml and 5 ng of human epidermal growth factor per ml (GIBCO BRL). Third to fifth passage cells were used.

#### 30 **Keratinocyte differentiation assays**

HaCaT or NHK cells were grown post confluence in the same medium as used for keratinocyte culture. Suspension culture was carried out as described (Watt, 1994). Briefly, after trypsinization, keratinocytes, were suspended in keratinocyte serum-free medium (10<sup>7</sup> per ml). This liquid suspension was then diluted (1:10) in keratinocyte

serum-free semisolid medium containing the aforementioned supplements and 2% methylcellulose (Aldrich, Bornem, Belgium) and incubated on 0.4% poly(2-hydroxyethylmethacrylate) (Sigma, St. Louis, MO) coated dishes.

Vitamin D treatment was performed in Keratinocyte Serum Free medium without insulin, thyroid hormone, and hydrocortisone because higher vitamin D anti-proliferative activity was described in the absence of insulin (Chen *et al*, 1995). Vitamin D was added from a stock solution of  $10^{-4}$  M per ml in absolute ethanol. 1,25-dihydroxyvitamin D<sub>3</sub> was purchased from Sigma Chemical Co. (St. Louis, MO). The cells were given fresh medium, containing the vitamin D, every 24 hours.

#### 10 ***Immunoblot analysis***

Transfected cells, differentiated keratinocytes or skin tissue were lysed in a buffer containing 1% NP-40, 200 mM NaCl, 10mM Tris HCl pH7-7.5, 5 mM EDTA, 10% glycerol, 1mM PMSF, 0.1 mM aprotinin and 1mM leupeptin. These lysates were immunoblotted with the indicated antibodies. Anti-keratin 10 and anti-human caspase-3 were purchased from Santa-Cruz and Biosource, respectively. Antibodies against murine caspases were raised by injecting rabbits with purified, bacterially expressed caspase preparations (Van de Craen *et al.*, 1999). Note that the anti-murine caspase-14 antibody cross-reacts with human caspase-14 on western blots as well as on immunohistochemistry staining.

#### 20 ***In vitro* transcription/translation**

pCDNA-plasmids containing mouse or human caspase-14 full length cDNA (pDNA1mCASP-14 and pCDNA3hCASP-14) were used to produce radiolabelled precursor caspase-14 by coupled transcription/translation (TNT kit from Pomega).

#### ***In vitro* caspase-14 cleavage assay**

25 2 microliter of *in vitro* radiolabelled caspase-14 was incubated with 5 µg of skin or cell lysates in a total volume of 25 µl caspase buffer (50 mM HEPES, pH7.5, 1mM EDTA, 10 mM DTT, 1 mM PMSF, 50 µM leupeptin and 20 µg/ml aprotinin) for 90 minutes at 37 °C. Skin and cell lysates were prepared in a buffer containing 1% NP-40, 200 mM NaCl, 10 mM Tris HCl pH 7.0 to 7.5, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 50 µM leupeptin and 20 µg/ml aprotinin. In case of addition of caspase inhibitors, these were used at a final concentration of 10 µM and pre-incubated with the lysates for 30 minutes at 37 °C. The resulting cleavage products were analysed on a 15% SDS-PAGE.

## Results

### **1) Overexpression of procaspase-14 does not induce cell death or autoprocessing.**

In previous experiments we showed that overexpression of murine caspase-14  
5 did not induce cell death (Van de Craen *et al.*, 1998a). To examine whether  
overexpression of human caspase-14 is able to induce cell death, HEK293T or MCF-7  
cells were transiently transfected with different vectors containing mouse or human  
procaspase-14. Since it has previously been shown that caspase-8 is capable of  
inducing apoptosis in both cell lines (Boldin *et al.* 1996; Muzio *et al.*, 1996), a caspase-  
10 8 expression vector was used as a positive control. A vector coding for GFP was co-  
transfected, which made it possible to visualise the transfected population by  
fluorescence microscopy and analyse whether the cells had the morphological  
features of apoptosis. In HEK293T cells or MCF-7 cells overexpression of caspase-8  
resulted respectively in 89% or 80% cell death 24h after transfection. (Fig. 1A).  
15 However, in neither of both cell lines transient overexpression of human caspase-14  
did result in any cell death. Removal of a putative 13 amino acids long prodomain  
contained in human caspase-14 did neither result in apoptosis.

Upon overexpression caspases do autoprocess, thereby generating the p10  
and p20 subunits, resulting in fully enzymatically active caspase eventually leading to  
20 cell death (Molineaux *et al.*, 1993; Kamens *et al.*, 1995; Van de Craen *et al.*, 1997 and  
1998b). By means of western blot analysis human caspase-14 processing was  
evaluated. Overexpression of caspase-14, nor with or without the putative prodomain,  
in MCF-7 or HEK293T did not result in processing (Fig. 1B). This in contrast to other  
short prodomain caspases such as caspase-3, -6 and -7 that show autoprocessing,  
25 resulting in the appearance of proteolytic fragments corresponding to large and small  
subunits, when overexpressed in HEK293T cells (Fig. 1C).

### **2) Caspase-14 expression is restricted to epithelia of ectodermal origin.**

Previously it was shown that the tissue distribution of caspase-14 mRNA is  
limited to the adult skin and embryo (Van de Craen *et al.*, 1998a). To further  
30 investigate where exactly caspase-14 is expressed, we performed  
immunohistochemistry on embryo sections, using a polyclonal anti-mouse-caspase-14  
antibody. As a negative control the stainings were performed with the anti-rabbit-HRP  
antibody only.

Immunostaining of 15.5 dpc embryo sections revealed that caspase-14 is expressed in the differentiated layers of the epidermis (Fig. 2A), in the pigmented layer of the retina and in the epithelial cells of the choroid plexus (Fig. 2B and C). Caspase-14 is not expressed in the embryonic skin periderm or in the basal keratinocyte layers (*stratum basale*), these layers represent undifferentiated keratinocytes and contain neither the typical differentiation markers of adult skin (Byrne *et al.*, 1994). Moreover, caspase-14 is expressed in the fully developed hair bulb that also consists of differentiated keratinocytes (Hardy, 1992), whereas in a premature hair bulb, consisting of only cells of the *stratum basale*, there is no caspase-14 expression (Fig. 2A).

In addition, the epithelial cells of the choroid plexus stained more strongly in 15.5 dpc embryos than in the 13.5 dpc embryo's. In the epithelial cells of the choroid plexus and the retinal pigment layer there is only cytoplasmic expression, while in skin also nuclear expression occurs.

### **3) Caspase-14 is gradually expressed in the differentiating skin**

Immunohistochemistry on human skin sections confirmed that the caspase-14 protein is only expressed in the differentiated layers of the epidermis but not in the basal layer (Fig. 3). The expression increases gradually in the skin from the *stratum spinosum* towards the *stratum granulosum* and the *stratum corneum*. During keratinocyte differentiation the caspase-14 expression also becomes more pronounced in the nucleus, especially in the *stratum granulosum*. In a later stage the nucleus gets expelled, thereby reaching the final stage of differentiation resulting in the formation of the cornified layers. The mature hairshaft and the sebaceous gland consist of differentiated keratinocytes (Hardy, 1992), and also here caspase-14 is expressed. In the sweat glands, which are of dermal origin, the staining was negative.

### **4) The expression of caspase-14 is drastically reduced in the skin plaques of psoriatic patients.**

Psoriasis is a common human disease with distinctive histological hallmarks including epidermal keratinocyte hyperproliferation, abnormal keratinocyte differentiation, parakeratosis and immune cell infiltration. Until now it is not clear if psoriasis is caused by a defect in the keratinocyte or the recruited immune cells. To evaluate a potential role for caspase-14 in skin pathologies, we analysed samples from psoriatic skin plaques by immunohistochemistry with anti-caspase-14 antibodies. Plaques from 6 independent psoriatic were analysed and compared to the same

number of non-involved patients. As is clear from Fig.3 (showing one representative patient from psoriatic and non-involved skin) the psoriatic plaques show a marked decrease in the expression of caspase-14. In the parakeratotic lesions and in the abnormal, multi-layered *stratum basale* caspase-14 expression was seemingly absent in case of psoriatic skin. Non-involved skin stained in a pattern as discussed above. These data show an involvement of caspase-14 in abnormal skin differentiation pathologies. Strategies to upregulate caspase-14 expression may prove to have an important therapeutic contribution to diseases involving parakeratosis, such as psoriasis.

10 **5) Caspase-14 is processed in normal mouse epidermis.**

As shown in Fig.1 we were unable to generate processed caspase-14 upon overexpression in HEK293T or MCF-7 cells. To analyse if caspase-14 is present in an activated state in developing skin, we made a protein extract of mouse skin and used these lysates for immunoblotting with different rabbit polyclonal anti-caspase antisera. In the case of caspase-14 we could clearly detect bands corresponding both to the proenzyme and to the processed p20 fragment (Fig. 4). The two bands that can be observed at the size of approximately 20 and 18.5 kDa probably represent the large subunit with and without prodomain, respectively. The specificity of these bands for caspase-14 was confirmed in a competition experiment in which the western blot was developed with anti-caspase-14 in the presence of recombinant, bacterially expressed caspase-14. Under these conditions any of the anti-caspase-14 stained fragments could be detected, in contrast to incubation with anti-caspase-14 in the presence of recombinant caspase-3. The typical apoptosis-related caspases such as caspase-3, -6 and -7 were only present as unprocessed proenzymes. Although, an involvement of active caspase-3 has been claimed in skin differentiation (Weil *et al.*, 1999). Since it is well documented that IL1 is produced in the skin (Kumar *et al.*, 1992; Boehm *et al.*, 1995; Debets *et al.*, 1995), we also analysed the activation state of caspase-1 and -11. It has been shown in physiological systems that these two caspases are involved in the processing and secretion of IL1- $\alpha$  and - $\beta$  (Kuida *et al.*, 1995; Li *et al.*, 1995; Li *et al.*, 1997; Wang *et al.*, 1998). As expected we could detect prototype bands correlating to processed forms of caspase-1 and -11.

**6) The expression of caspase-14 in human keratinocytes is inducible in vitro.**

Since caspase-14 is not present in the *stratum basale* but is expressed in the differentiated layers of the epidermis, we wondered whether the protein expression of caspase-14 in keratinocytes could be induced *in vitro* by conditions of differentiation. In a western blot experiment it was shown that cell lysates of undifferentiated HaCaT cells or normal human keratinocytes do not contain caspase-14 (Fig. 5A), which was expected since these undifferentiated cells represent the *stratum basale* cells of the epidermis where no caspase-14 is expressed.

Primary human keratinocytes grown in suspension or post confluence express several differentiation markers such as keratin 1 and 10, involucrin and transglutaminase (Poumay and Pittelkow, 1995). We could show that in cells grown under these differentiating conditions caspase-14 was expressed together with keratin 10 (Fig. 5A and B). In contrast, caspase-3 expression was hardly affected when cells were grown post confluence. These differentiation conditions did not lead to the processing of caspase-3 or -14. As a control for caspase-3 processing lysates were loaded derived from U937 cells treated with TNF and cycloheximide.

Treatment of keratinocytes with 1,25-dihydroxyvitamin D<sub>3</sub> also induces these cells to differentiate (Hosomi *et al.*, 1983; Bikle and Pillai, 1993; Chen *et al.*, 1995). HaCat keratinocytes were treated with different concentrations of vitamin D and cells were lysed 72h after treatment. We were able to show that vitamin D is capable of inducing the expression of procaspase-14 in a dose-dependent way, reaching a maximal level at 10<sup>-7</sup> to 10<sup>-6</sup> M (Fig. 6A). This concentration range of vitamin D has also been described as the optimal concentrations for vitamin D-induced anti-proliferative effects on keratinocytes (Simpson *et al.*, 1980; Segaert *et al.*, 1997). The non-treated cells also express low levels of procaspase-14 due to post confluent growth of the cells at this time-point. Moreover, keratin 10 was expressed in these cultures, indicating that differentiation was already occurring. Vitamin D treatment does not enhance the expression of keratin 10, however apparently its expression could no longer be detected when vitamin D was added at a concentration of 10<sup>-6</sup> M. It is possible that at this concentration keratinocyte differentiation reaches its final stage, eventually leading to the formation of keratin complexes that are not solubilised in classical lysis buffers (Eichner *et al.*, 1989 and 1990). To analyse the vitamin D-induced caspase-14 expression in time, we performed a kinetics experiment in which HaCat cells treated with a fixed concentration of 1,25-dihydroxyvitamin D<sub>3</sub> (10<sup>-7</sup>M) and lysed after 24, 48, 72 and 96 hours (Fig. 6B). Caspase-14 was increasingly expressed

from 48 hours on, to 96 hours treatment. Background expression of caspase-14 increased in time due to the post confluent differentiation state of the cells, as shown by keratin 10 expression. In neither condition we were able to observe fragments of caspase-14 pointing to processing of caspase-14, as was observed in mouse skin lysates (Fig. 4). Again, the caspase-3 expression or processing level was not affected by treatment with vitamin D, or by post confluent growth. This in contrast to the proposed role of activated caspase-3 in keratinocyte differentiation (Weil *et al.*, 1999).

#### **7) Caspase-14 is activated during epidermal differentiation**

To determine the kinetics of caspase-14 activation during epidermal differentiation we monitored the appearance of its cleavage products during the formation of epidermal equivalents (EE, i.e. in vitro reconstituted epidermis purchased from Mattek Corp Ashland MA) EE consists of several layers of differentiating KC and form a stratum corneum comparable to that of normal human epidermis *in vivo* and can therefore be considered an *in vitro* model for terminal differentiation of KC. For the analysis of the time course of caspase-14 processing, EE were either left submerged in culture medium or lifted to the air-liquid interface after onset of stratification. Samples obtained at different timepoints were stored frozen on dry ice, until further analysis.

Western blot analysis for caspase-14 was performed with lysates of EE. Specificity of the antibody was controlled by pre-incubation with recombinant caspase-14. Kinetics of caspase-14 cleavage was studied by analysing EE at 2, 4 and 7 days after lifting at the air liquid interface. In parallel EE maintained submerged in culture medium were tested. Morphology of submerged EE and EE lifter to the interface was analysed by hematoxylin eosin staining of paraffin sections.

For Western blot analysis all samples were lysed in SDS-lysis buffer (62.5 mM Tris/HCl, pH 6.8, 6M urea, 2% SDS, 0.00125% bromophenolblue, 5%  $\beta$ -mercaptoethanol). After ultrasonification and removal of insoluble cell debris, 40 $\mu$ g of protein were electrophoresed through a 8% to 18% gradient polyacrylamide gel and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were blocked for 3 h in blocking buffer (PBS with 7.5% nonfat dry milk, 2% BSA, 0.1% Tween) and incubated with caspase-14 specific antiserum diluted 1:1000 in blocking buffer overnight at 4°C. Subsequently, membranes were washed in PBS with 5% nonfat dry milk, 0.1% Tween, incubated with peroxidase-conjugated goat anti-rabbit IgG Fc antibody (Pierce, 1:10000 in blocking buffer) for 1h at room temperature, washed in

PBS and developed using the ECL chemiluminescent detection system (Amersham). As control the anti-caspase-14 antiserum was preincubated with 10 µg/ml recombinant mouse caspase-14 for 1 hour at room temperature.

Thus, in this system, the exposure of the culture to air is a strong stimulus for the formation of a regular epidermis (Asselineau et al., 1985). Therefore we compared EE cultured at the air-liquid interface with EE left submerged in the medium. Under submerged conditions, EE failed to differentiate and did not form a regular stratified epidermis. After 7 days the uppermost KC layers still contained nuclei. Although high amounts of the caspase-14 proenzyme (28kD) were detectable via Western blot at all time points, only minute amounts of caspase-14 cleavage products (11 and 17kD) could be detected after 7 days. By contrast, in EE which were lifted at the air liquid interface, KC differentiation proceeded to the formation of a well structured stratum corneum, devoid of nuclei. In lysates of these EE, subunit bands of caspase-14 demonstrating its activation were present already two days after lifting EE to the air-liquid interface and strong processing was observed on day 7.

#### **8) Regulation of caspase-14 expression by vitamin D treatment of psoriatic skin.**

Skin samples were derived from psoriatic patients. Psoriatic plaques were treated with calcipotriol-Daivonex. The treatment with calcipotriol was two times per day and was applied in a salve. Non-involved skin from the same patients served as a control. Biopsies of the psoriatic plaques were taken before and after treatment. Skin samples were fixed, paraffin embedded and sliced. Caspase-14 protein expression was detected by means of immunohistochemistry using a polyclonal anti-caspase-14 antibody. The vitamin D treatment clearly results in an increased expression of caspase-14 in all suprabasal layers, including the parakeratotic plaques. Both nuclear and cytoplasmic staining was increased by the treatment. Also the thickening of the psoriatic skin is significantly reduced by treatment with vitamin D. Seven patients were treated with vitamin D and analysed for caspase-14 expression. All patients gave similar results (see table 1).

#### **9) Caspase-14 expression is reduced in papillomas and carcinomas of the choroid plexus**

We analyzed caspase-14 expression by means of immunohistochemistry in human papillomas and carcinomas of the choroid plexus and compared this expression to normal levels of expression in the epithelium of the choroid plexus. Caspase-14 expression could be demonstrated in the epithelial cells of normal choroid plexus.

whereas caspase-14 expression in both papilloma and carcinoma of the choroid plexus was strongly reduced (see Figure 7). These tumors share with the normal choroid plexus the property of cerebrospinal fluid (CSF) production. Overproduction of CSF by these tumours is a well-recognized phenomenon, eventually leading to hydrocephalus. Since a reduced caspase-14 expression level correlates with a dysfunctional choroid plexus epithelium, increasing caspase-14 expression by use of caspase-14 expression modulators or by gene therapy restores normal functioning of the choroid plexus.

**10) Regulation of caspase-14 expression in keratinocyte cultures by vitamine D, retinoic acid, lithium salts and glucocorticoids**

Induction of caspase-14 protein expression was further analyzed upon treatment of HaCaT cells with vitamin D<sub>3</sub>, retinoic acids, LiCl, cytokines or glucocorticoids. Before treatment the cells were grown subconfluent or induced to differentiate and express procaspase-14 by means of post-confluent growth. The cells that were grown subconfluent do not express caspase-14. However, the expression of procaspase-14 could be induced by treatment of the cells with vitamin D<sub>3</sub>, LiCl or hydrocortisone. In cells grown post-confluent the caspase-14 expression level augmented upon treatment with vitamin D<sub>3</sub>, LiCl or hydrocortisone and was lower after treatment with retinoic acids. Both 9-*cis* retinoic acid and all-*trans* retinoic acid were used. All-*trans* retinoic acid at concentrations less than 10<sup>-6</sup>M and 9-*cis* retinoic acid have been described as antagonists for vitamin D dependent antiproliferative activity, while at pharmacologic concentrations (10<sup>-6</sup>M) all-*trans* retinoic acid can potentiate this effect. In the sub- or post-confluent grown cells the vitamin D<sub>3</sub> inducing effect on caspase-14 expression could be inhibited by addition of retinoic acids. At concentrations lower than 10<sup>-6</sup>M all-*trans* retinoic acid, there was less inhibition on the vitamin D dependent induction of caspase-14 expression. Cytokines (TNF, IL-1 beta, IFN-gamma) did not influence caspase-14 expression, although the inducing effect of LiCl was inhibited in combination with TNF (see Fig. 8).

**11) Modulation of caspase-14 via nucleic acids encoding for caspase-14 affects skin differentiation**

The effect on skin differentiation of overexpression or downregulation of caspase-14 is monitored *in vitro*. Therefore retroviral expression vectors are developed containing wild-type caspase-14 (over-expression), a catalytically inactive caspase-14 mutant that could act as a dominant-negative form of caspase-14 (downregulation) or an antisense

caspase-14 sequence (downregulation). The catalytically inactive mutant is obtained by modifying the catalytic important residues such as His89 or Cys132 (referring to the human caspase-14 sequence as previously referred to) to Ala. Examples of retroviral vectors used for transfection of keratinocytes are EBV-based vectors such as the LZRS retroviral vector (Seitz et al., 1998) or pBabe puro (Lowell et al., 2000). These vectors are then used to transfect primary keratinocyte cultures with an > 99 % transfection efficiency. The transfected keratinocytes are then used in an *in vitro* epidermal differentiation assay and the effect of upregulation or downregulation of caspase-14 on the outcome of the epidermal differentiation is monitored by means of histological analysis and western blotting. The western blotting indicates to what level caspase-14 is upregulated/down-regulated and processed when compared to control cultures.

## **12) Skin lysates contain a caspase-14 processing activity**

In vitro translated and radioactive <sup>35</sup>S-methionine labelled murine or human procaspase-14 was incubated with lysates of undifferentiated or differentiated keratinocytes (HaCaT or NHK cells), or lysates of mouse skin (C57BL/6 mice) for 90 minutes at 37°C and analysed by means of SDS-PAGE and autoradiography. The differentiated keratinocytes were obtained by growing these cells postconfluence; this condition was shown to upregulate caspase-14 expression but not processing. Both mouse and human caspase-14 were processed to its catalytic subunits p20 and p10 by an enzymatic activity present in the mouse C57BL/6 skin lysate. This activity was neither detectable in lysates of undifferentiated nor in vitro differentiated (postconfluence = pc) keratinocytes. These data correlate with our observations that in skin caspase-14 is present in its processed form and that postconfluence grown keratinocytes only show procaspase-14 expression. To test if a known caspase-activity could be responsible for the caspase-14 processing activity present in skin lysates, we incubated these lysates with different peptide inhibitors before co-incubation with the labelled procaspase-14 for 30 minutes at 37 °C. None of the used inhibitors affected the procaspase-14 processing activity present in skin lysates. We used the well-known caspase inhibitors zVAD, BocD, DEVD and YVAD and the cathepsin B inhibitor zFA.

## Tables:

treatment	Patient (sample number)	stratum basale		stratum spinosum		stratum granulosum		stratum granulosum beneath parakeratotic		stratum corneum				epidermis thickness (µm)		
		cytoplasm	nucleus	cytoplasm	nucleus	cytoplasm	nucleus	cytoplasm	nucleus	parakeratotic layer		orthokeratotic layer		mean	max	min
1	A (PS 101/1)	-	-	-	+	+	++					-/+		87	115	51
1	B (PS 103/2)	-	-	+	+	+	++					+		94	109	74
1	C (PS 104/2)	-	-	+	+	+	++					+		75	109	46
1	D (PS 105/2)	-	-	-/+	+	+	++					+		111	153	80
1	E (PS 106/2)	-	-	+	+	+	++					+		117	140	86
1	F (PS 107/1)	-	-	+	+	+	++					+		87	109	68
1	G (PS 110/2)	-	-	+	+	+	+					+		126	197	90
2	A (PS 101/2)	-	-	-/+	-/+	-/+	+	-	-/+	-/+	-			283	401	193
2	B (PS 103/1)	-	-	-	-/+	-/+	+	-	-	-	-			389	485	283
2	C (PS 104/1)	-	-	-/+	+	+	++	-/+	+	-	-			241	285	165
2	D (PS 105/1)	-	-	-	+	-/+	+	-/+	+	-/+	-/+			377	463	251
2	E (PS 106/1)	-	-	-	-/+	-/+	+	-/+	+	-/+	-/+			430	529	313
2	F (PS 107/2)	-	-	-	-/+	-/+	+	-/+	+	-/+	-/+			541	628	507
2	G (PS 110/1)	-	-	-	-/+	-/+	+	-/+	+	-/+	-/+			364	456	239
3	A (PS 102)	-	-	-/+	+	+	++	+	+	+	+			185	234	145
3	B (PS 108)	-	-	-/+	+	+	++			-/+	-/+			264	358	179
3	C (PS 109)	-	-	-/+	+	+	++	+	+			+		187	250	131
3	D (PS 112)	-	-	+	+	+	++	+	+	+	+			247	302	179
3	E (PS 113)	-	-	-/+	+	+	++	+	+	-/+	-/+			326	371	278
3	F (PS 114)	-	-	-/+	+	+	++	+	+	-/+	-/+			284	375	158
3	G (PS 115)	ND														

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### Claims

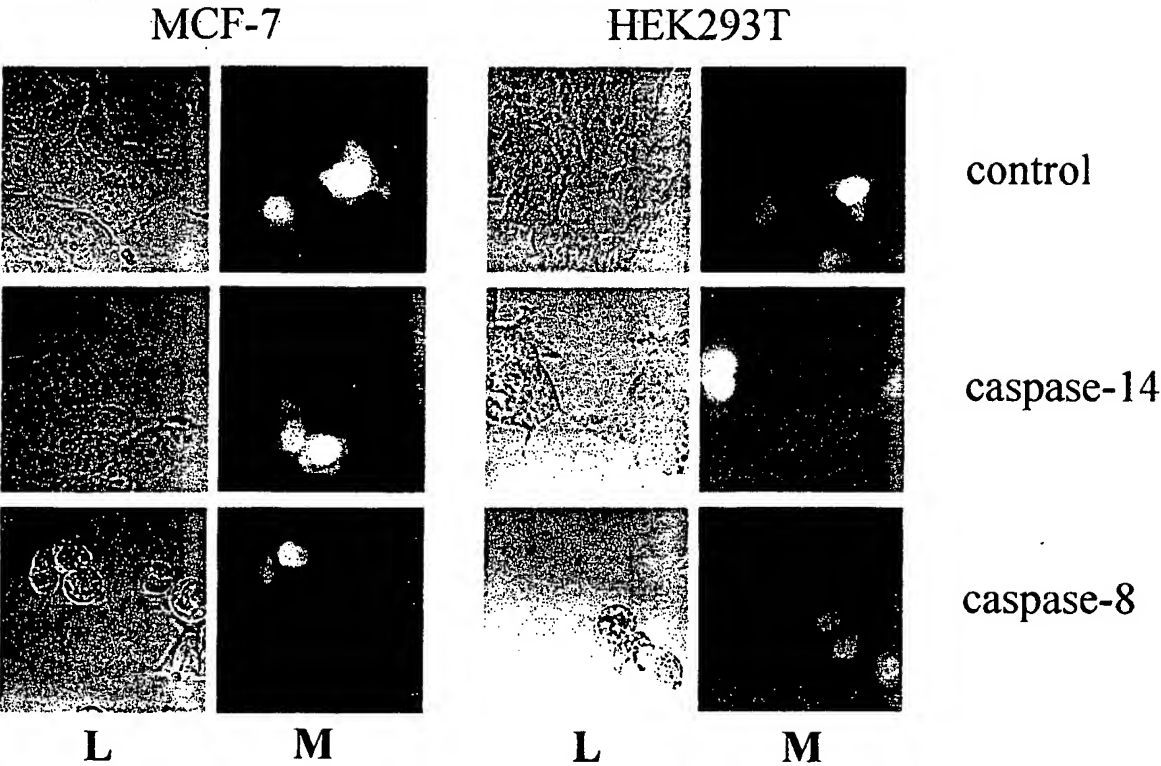
1. Use of modulators of caspase-14 bioactivity, nucleic acids encoding caspase-14 and/or caspase-14 protein for the manufacture of a medicament that modulates keratinocyte differentiation and/or the blood-eye barrier and/or the blood-brain barrier.
2. Use according to claim 1 wherein said modulators are chosen from the group comprising modulators of caspase-14 expression, modulators of caspase-14 activation and/or bioactivity, and/or fragments of said modulators that modulate keratinocyte differentiation and/or the blood-eye barrier and/or the blood-brain barrier.
3. Use according to claim 2, wherein said modulators of caspase-14 activation and/or bioactivity are modulators that bind to caspase-14 protein.
4. Use according to claim 3, wherein said modulators that bind to caspase-14 protein are antibodies and/or fragments thereof that modulate keratinocyte differentiation and/or the blood-eye barrier and/or the blood-brain barrier.
5. Use according to claim 2, wherein said modulators of caspase-14 expression are chosen from the group comprising anti-sense nucleic acids that hybridize with nucleic acids encoding caspase-14 or fragments thereof, ribozymes, vitamine D or homologues thereof, retinoic acid or homologues thereof, transformation growth factor-beta or homologues thereof, glucocorticoids and homologues thereof, lithium salts and calcium salts.
6. Use according to claim 5 wherein said vitamine D is vitamine D<sub>3</sub>, wherein said glucocorticoid is hydrocortisone and wherein said lithium salt is lithiumchloride.
7. Use of modulators of caspase-14 bioactivity, nucleic acids encoding caspase-14 and/or caspase-14 protein for the manufacture of a medicament for treating disorders of keratinocyte differentiation, disturbances in the blood-eye barrier and brain disorders.
8. Use of nucleic acids hybridising with nucleic acids encoding for caspase-14 or fragments thereof, or, molecules specifically binding to caspase-14 protein and/or fragments thereof for the manufacture of a diagnostic assay for disorders of keratinocyte differentiation, disturbances in the blood-eye barrier and/or disturbances in the blood-brain barrier.
9. A pharmaceutical composition comprising modulators of caspase-14 bioactivity,

nucleic acids encoding caspase-14 and/or caspase-14 protein.

10. A pharmaceutical composition according to claim 9 wherein said modulators  
are chosen from the group comprising modulators that bind to caspase-14  
protein, anti-sense nucleic acids that hybridize with nucleic acids encoding  
caspase-14 or fragments thereof, ribozymes, vitamine D or homologues  
thereof, retinoic acid or homologues thereof, transforming growth factor-beta or  
homologues thereof, glucocorticoids and homologues thereof, lithium salts and  
calcium salts.

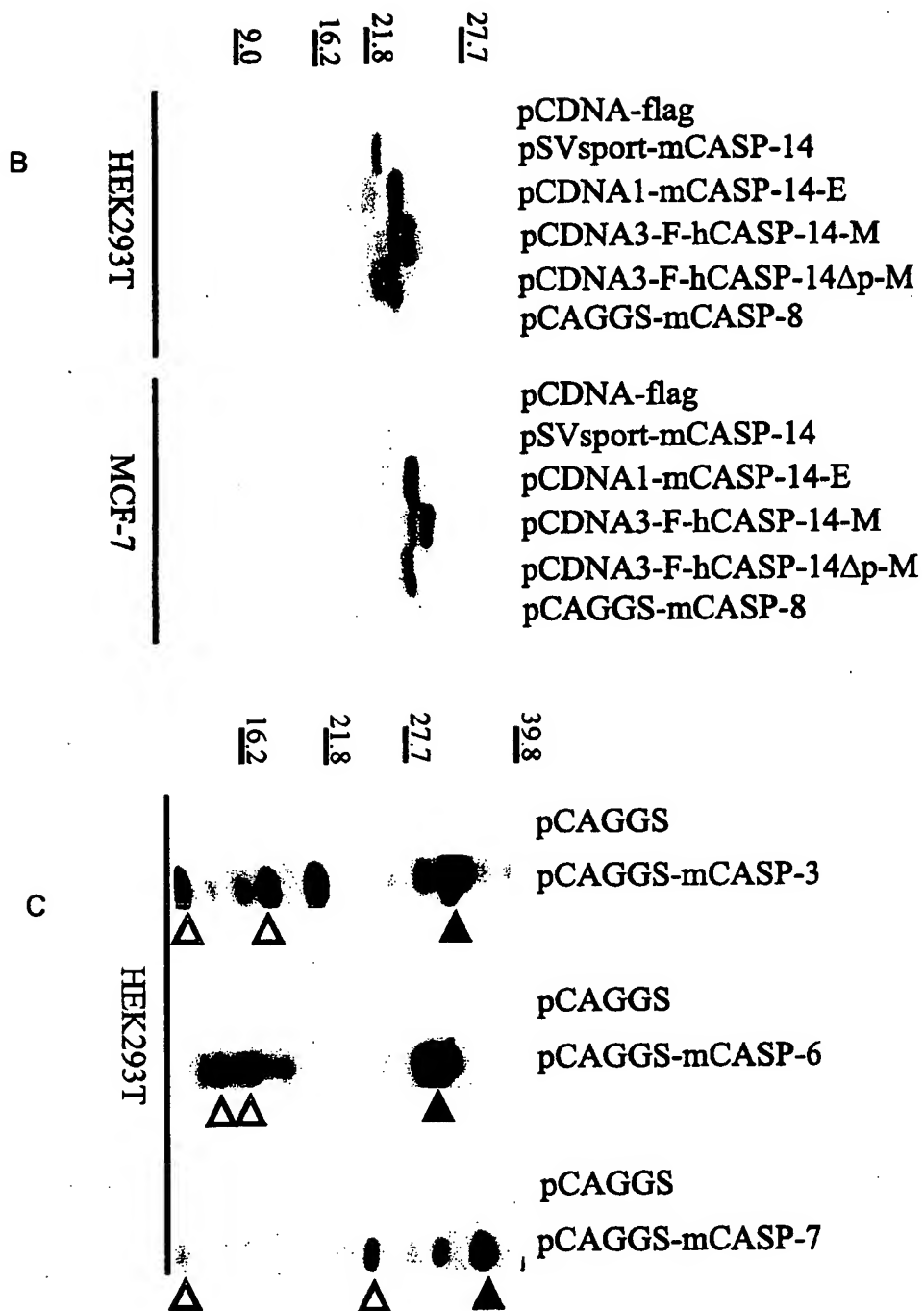
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Fig. 1A



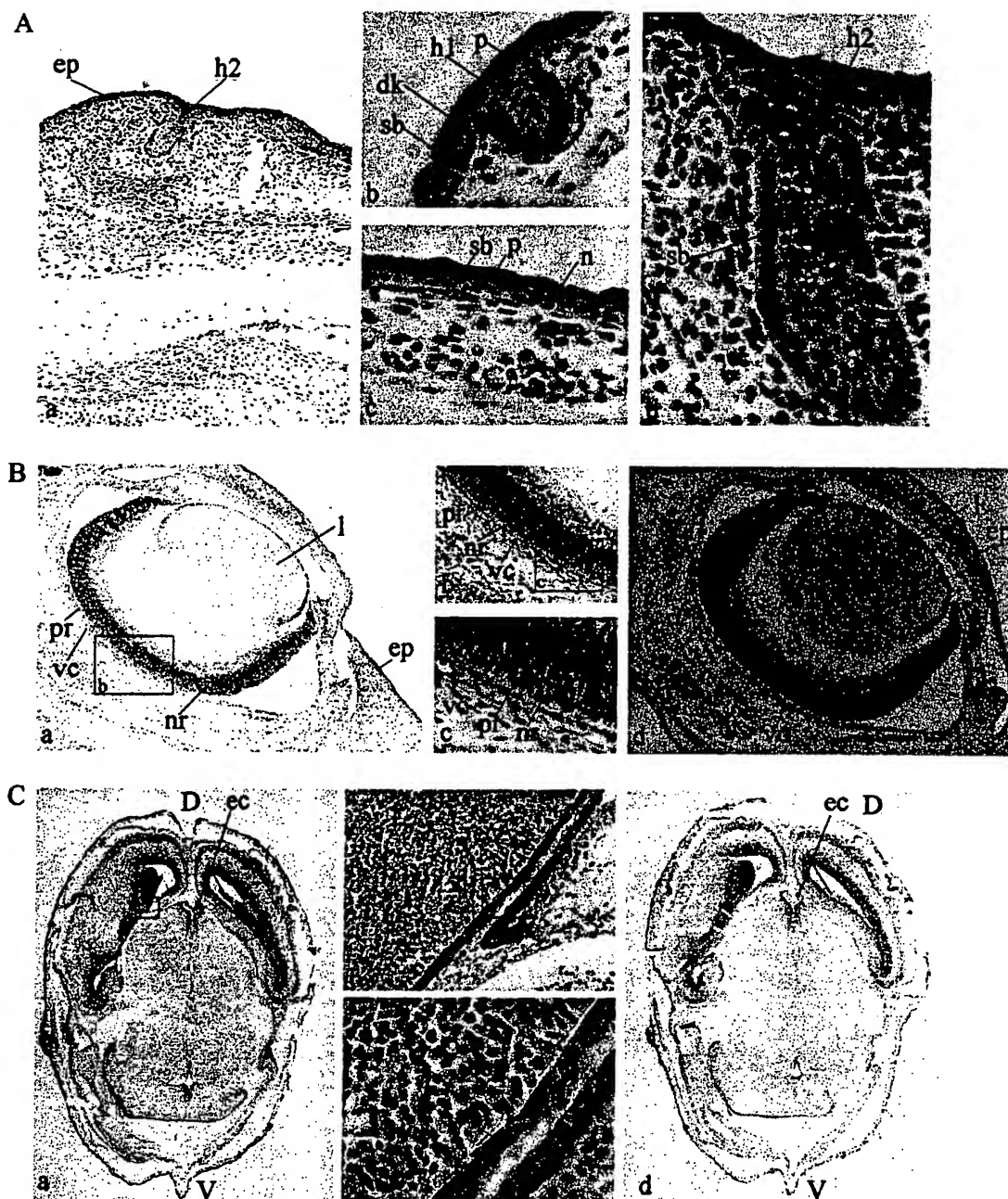
2/9

Fig. 1B and C



3/9

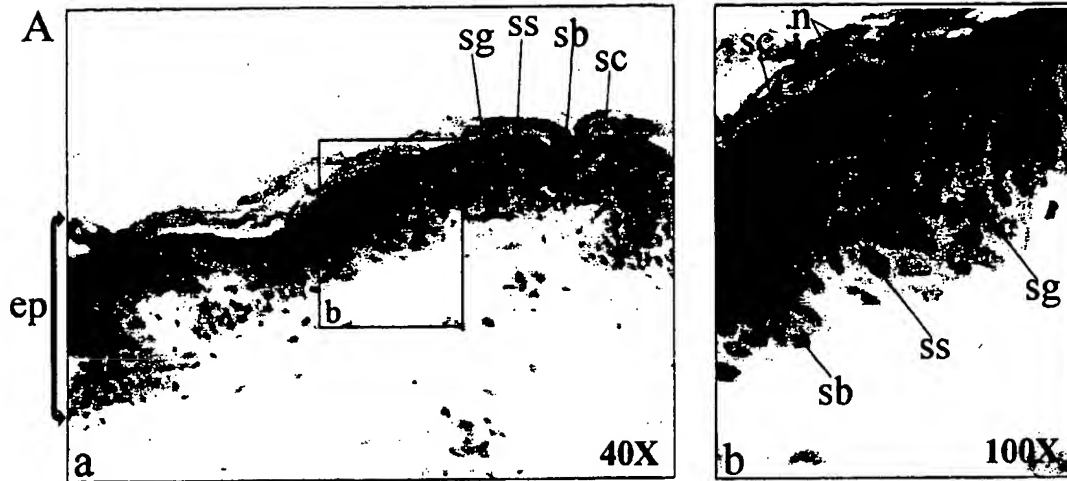
Fig. 2



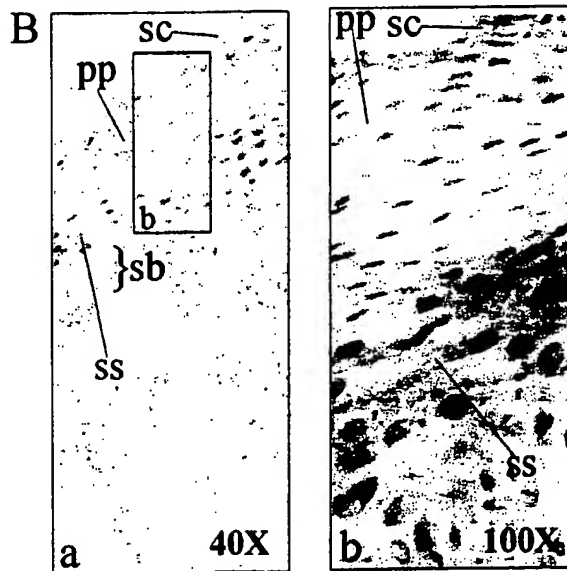
4/9

Fig. 3

Normal skin

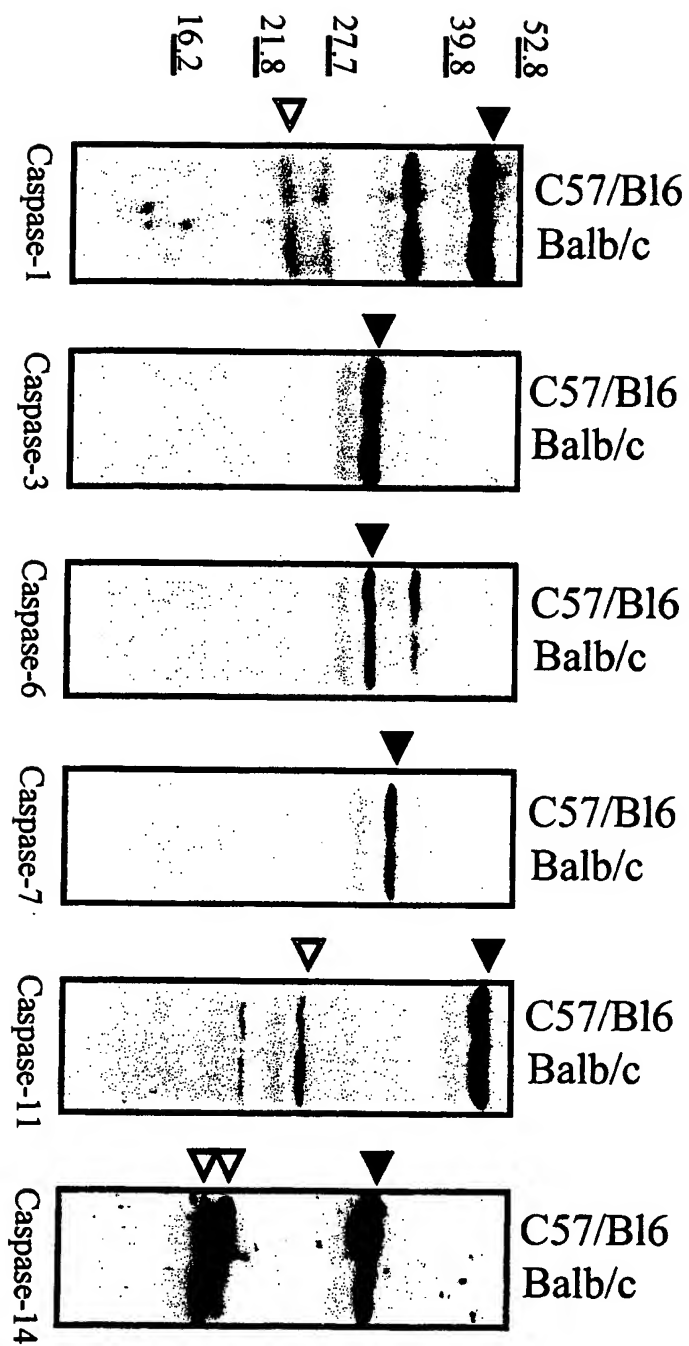


Psoriatic skin



5/9

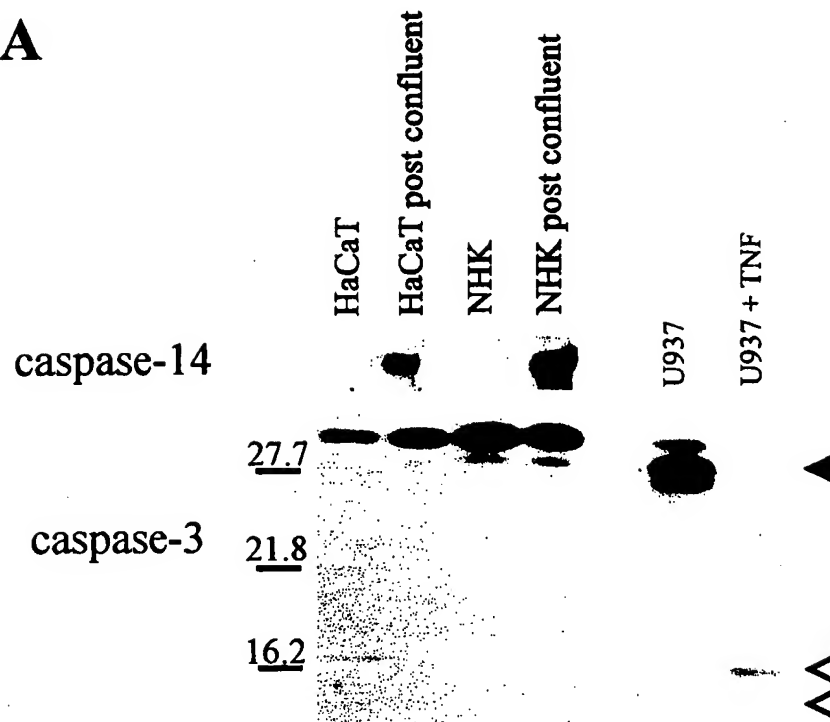
Fig. 4



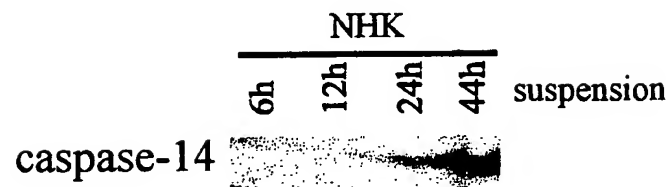
6/9

Fig. 5

**A**



**B**



**Fig. 6**

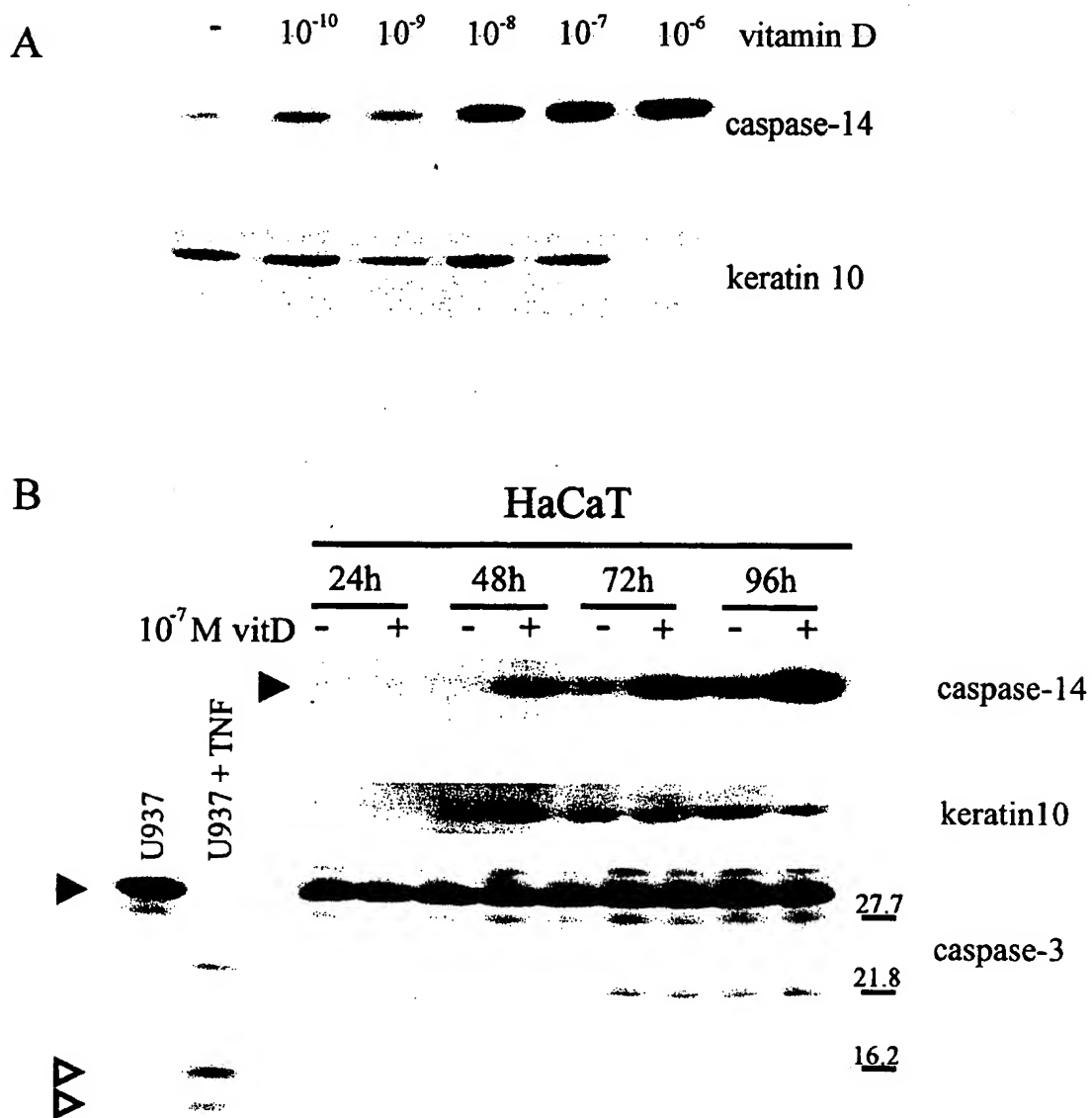
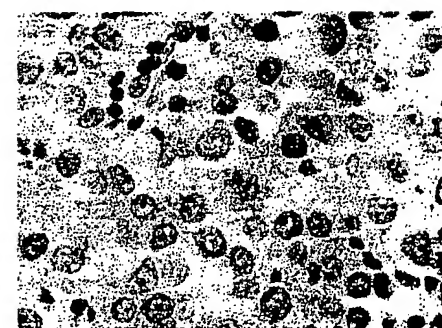
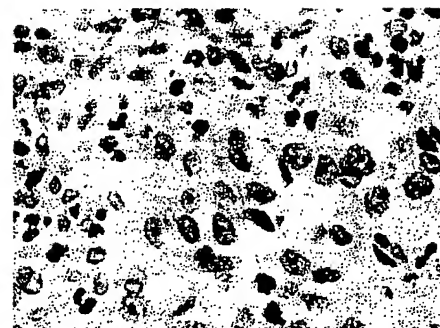
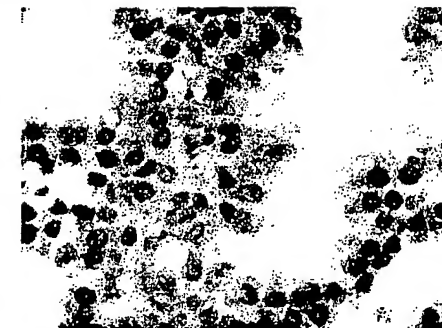
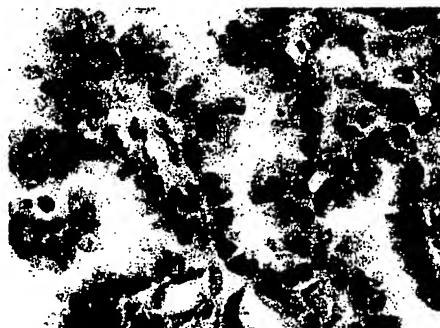
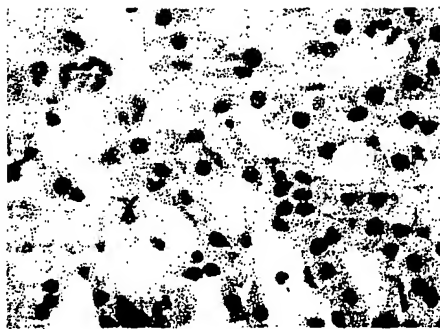
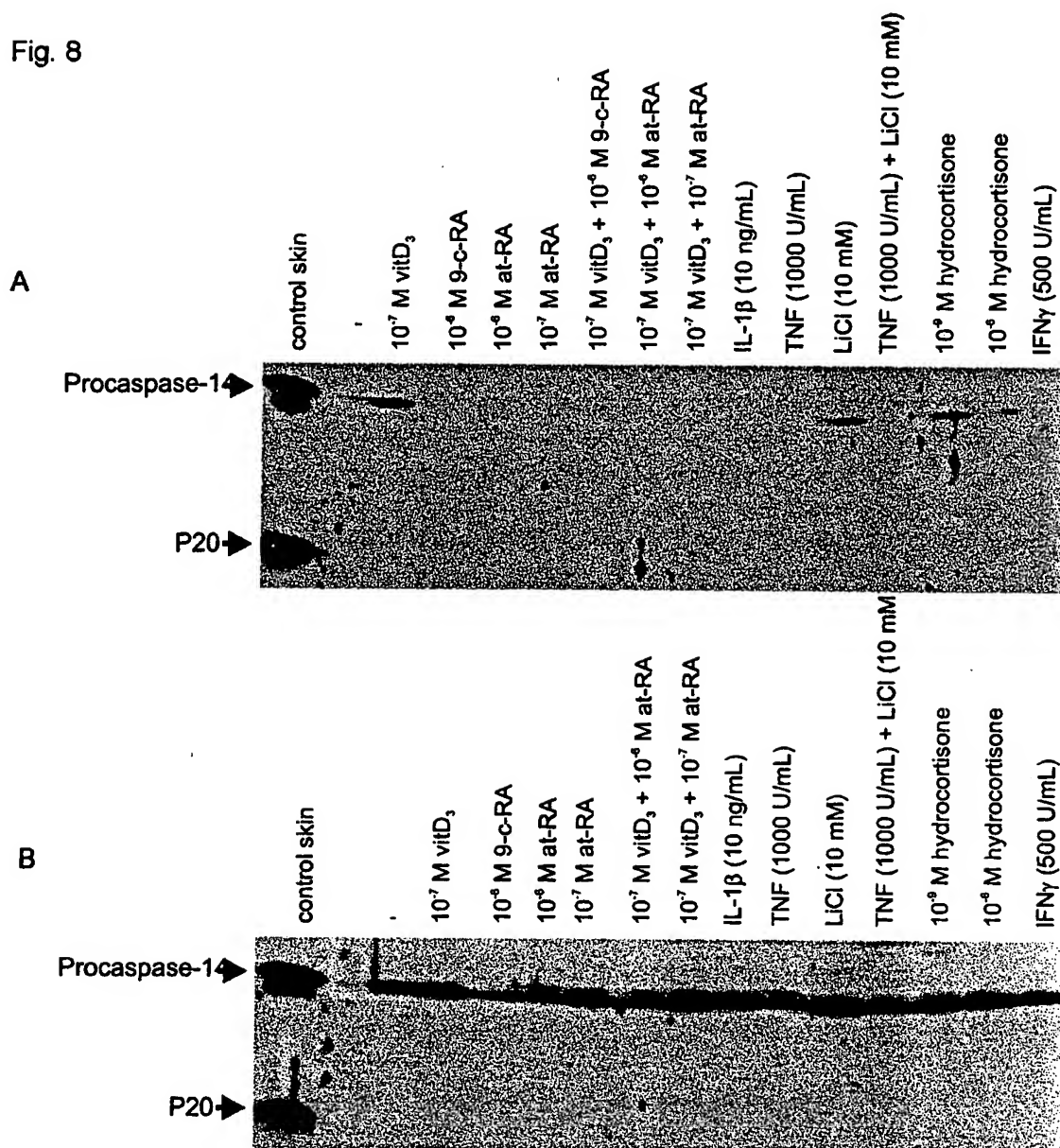


Fig. 7



9/9

Fig. 8



## SEQUENCE LISTING

<110> VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOL

<120> USE OF CASPASE-14 AND CASPASE-14 MODULATORS TO DIAGNOSE  
AND/OR TREAT SKIN, EYE AND BRAIN DISORDERS

<130> WDE/C14/V046

<140>

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<150> 99203752.3

<151> 1999-11-10

<150> 00202776.1

<151> 2000-08-04

<160> 3

<170> PatentIn Ver. 2.1

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: forward PCR  
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ataagaatgc ggccgcaagc aatccgcggt ctttggaaga ggag

44

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<212> DNA

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<223> Description of Artificial Sequence: forward PCR  
delta p primer

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<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: reverse PCR  
primer

<400> 3

gctagctagc ctgcagatac agccgtttcc ggag

34

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
17 May 2001 (17.05.2001)

PCT

(10) International Publication Number  
**WO 01/34183 A3**

- (51) International Patent Classification<sup>7</sup>: A61K 38/48, 39/395, 31/7088, 31/593, 31/203, 38/18, 31/573, 33/14, 33/06, C12Q 1/68, A61P 17/00, 27/00, 25/00
- (74) Common Representative: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).
- (21) International Application Number: PCT/EP00/11251
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date:  
10 November 2000 (10.11.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
99203752.3 10 November 1999 (10.11.1999) EP  
00202776.1 4 August 2000 (04.08.2000) EP
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (*for all designated States except US*): VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW [BE/BE]; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).
- Published:  
— with international search report
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): DECLERCQ, Wim [BE/BE]; Pieter Casierstraat 7, B-8510 Marke (BE). VANDENABEELE, Peter [BE/BE]; Nijverheidsstraat 18, B-9040 Sint-Amandsberg (BE). LIPPENS, Saskia [BE/BE]; Citadellaan 75, B-9000 Gent (BE).
- (88) Date of publication of the international search report:  
8 November 2001
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 01/34183 A3

(54) Title: USE OF CASPASE-14 AND CASPASE-14 MODULATORS TO DIAGNOSE AND/OR TREAT SKIN, EYE AND BRAIN DISORDERS

(57) Abstract: The present invention is based on the finding that caspase-14 is expressed in epithelial tissue such as the epidermis, the epithelial layer of the choroid plexus and the pigmented retinal layer. The invention relates to the involvement of caspase-14 in keratinocyte differentiation and in a properly functioning blood-brain barrier and blood-eye barrier. The present invention specifically relates to nucleic acids encoding caspase-14, caspase-14 protein and modulators of caspase-14 expression, activation and bioactivity that can be used to diagnose and/or treat keratinocyte differentiation disorders, disturbances in the blood-eye barrier and brain disorders.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/11251

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/48 A61K39/395 A61K31/7088 A61K31/593 A61K31/203  
 A61K38/18 A61K31/573 A61K33/14 A61K33/06 C12Q1/68  
 A61P17/00 A61P27/00 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 10504 A (ALNEMRI EMAD S ;FERNANDES ALNEMRI TERESA (US); IDUN PHARMACEUTICAL) 4 March 1999 (1999-03-04) page 4, line 11 - line 23 page 26, line 3 -page 30, line 19 page 35, line 19 - line 29	7-10
A	---	1-6
X	WO 99 23106 A (HUMAN GENOME SCIENCES INC ;NI JIAN (US); RUBEN STEVEN M (US)) 14 May 1999 (1999-05-14) page 3, line 30 - line 35 page 6, line 11 - line 28 page 20, line 17 - line 21 page 22, line 18 -page 24, line 9 ---	7-10
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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\*&\* document member of the same patent family

Date of the actual completion of the international search

22 June 2001

Date of mailing of the international search report

04/07/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Stein, A

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/11251

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CRAEN VAN DE M ET AL: "IDENTIFICATION OF A NEW CASPASE HOMOLOGUE: CASPASE-14" CELL DEATH AND DIFFERENTIATION, GB, EDWARD ARNOLD, OXFORD, vol. 5, no. 10, 1998, pages 838-847, XP000877350 ISSN: 1350-9047 cited in the application the whole document ---	1-10
X	WO 92 13551 A (ONCOGENE SCIENCE INC) 20 August 1992 (1992-08-20) page 6, line 21 - line 30 page 11, line 30 - page 12, line 14 claims 18-22 ---	1, 2, 5, 9, 10
X	EP 0 353 772 A (ONCOGEN) 7 February 1990 (1990-02-07) page 2, line 5 - page 3, line 21 page 5, line 38 - line 39 claims 1-10; examples 1, 3 ---	1, 2, 5, 7, 9, 10
X	EP 0 803 248 A (UNILEVER PLC ; UNILEVER NV (NL)) 29 October 1997 (1997-10-29) page 2, line 9 - line 23 page 3, line 1 - line 33 page 5, line 46 - line 52 example 1 page 20, line 17 - line 27 claim 9 ---	1, 2, 5, 7, 9, 10
X	US 5 037 816 A (HOLICK MICHAEL F ET AL) 6 August 1991 (1991-08-06) column 2, line 49 - column 3, line 7 column 4, line 32 - line 34 column 6, line 39 - line 52 claim 1 ---	1, 2, 5-7, 9, 10
X	EP 0 305 097 A (EFAMOL HOLDINGS) 1 March 1989 (1989-03-01) page 3, line 15 - line 25 page 4, line 34 - line 47 claims 1-14 ---	1, 2, 5-7, 9, 10
X	FR 2 726 187 A (THOREL JEAN NOEL) 3 May 1996 (1996-05-03) the whole document, especially page 2 lines 15-17 --- -/--	1, 2, 5, 7, 9, 10

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/11251

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS 'Online!  BIOSCIENCES INFORMATION SERVICE,  PHILADELPHIA, PA, US; 1990  KUNIYUKI S: "EFFECTS OF RETINOID 1-ALPHA  25 DIHYDROXYVITAMIN D-3 AND CORTICOSTEROID  ON THE CULTURED HUMAN EPIDERMAL  KERATINOCYTES LIGHT AND ELECTRON  MICROSCOPIC STUDY"  Database accession no. PREV199192088291  XP002170338  abstract  &amp; JOURNAL OF THE OSAKA CITY MEDICAL  CENTER,  vol. 39, no. 3, 1990, pages 553-574,  ISSN: 0386-4103</p> <p>---</p>	1,2,5-7, 9,10
A	<p>KAYA M ET AL: "Chemical induction of  fenestrae in vessels of the blood-brain  barrier."  EXPERIMENTAL NEUROLOGY,  vol. 142, no. 1, 1996, pages 6-13,  XP001009858  ISSN: 0014-4886  the whole document</p> <p>---</p>	2,5,7,9, 10
A	<p>AWASTHI P K ET AL: "Effect of oral  lithium on the action of various C.N.S.  active drugs."  INDIAN JOURNAL OF PHYSIOLOGY AND  PHARMACOLOGY,  vol. 40, no. 3, 1996, pages 241-244,  XP001009815  ISSN: 0019-5499  the whole document</p> <p>---</p>	1,2,5,7, 9,10
A	<p>HOHEISEL DIRK ET AL: "Hydrocortisone  reinforces the blood-brain barrier  properties in a serum free cell culture  system."  BIOCHEMICAL AND BIOPHYSICAL RESEARCH  COMMUNICATIONS,  vol. 244, no. 1,  6 March 1998 (1998-03-06), pages 312-316,  XP002170337  ISSN: 0006-291X  the whole document</p> <p>---</p>	1,2,5-7, 9,10
	<p>---</p> <p>-/--</p>	

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/11251

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>WO 00 04169 A (CRAEN MARC VAN DE ;VLAAMS  INTERUNIV INST BIOTECH (BE); DECLERCQ WI)  27 January 2000 (2000-01-27)  cited in the application  page 2, line 10 - line 24  page 3, line 14 - line 22  page 11, line 7 - line 19  page 13, line 7 - line 9  page 18, line 8 - line 15  claims 1-4,9-12  -----</p>	1-10

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-3,5,7,9,10 (all partially)

Present claims 1-3,7,9 relate to compounds defined by reference to a desirable property, namely their modulating effect on keratinocyte differentiation, on blood-eye-barrier and on blood-brain-barrier. However these claims do not contain any structural or essential characteristics of the compounds.

The claims cover all compounds having this property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds mentioned in the description at page 4 lines 2-7, page 8 lines 22-29, page 29 line 16- page 30 line 7, page 31 lines 16-28 and page 32 lines 15-26.

Additionally claims 5 and 10 relate to glucocorticoids in general having the effect as stated above. Because of the same reasoning as for the modulating compounds the search has been carried out with restriction to the glucocorticoid hydrocortisone as is disclosed on page 32 lines 15-26 and in claim 6.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/11251

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9910504 A	04-03-1999	AU 9121998 A EP 1012306 A	16-03-1999 28-06-2000
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EP 0305097 A	01-03-1989	AT 65182 T AT 96327 T AU 618730 B AU 1536188 A AU 2147988 A CA 1306944 A CA 1332358 A DE 3863678 D DE 3885212 D DE 3885212 T DK 225588 A DK 469488 A EP 0289204 A EP 0432700 A ES 2040847 T GR 3002426 T HK 127793 A IE 60568 B IE 61750 B JP 1013021 A JP 2699083 B JP 1083021 A KR 9613433 B KR 9700043 B NZ 224380 A NZ 225909 A SG 113593 G US 5252333 A	15-08-1991 15-11-1993 09-01-1992 27-10-1988 02-03-1989 01-09-1992 11-10-1994 22-08-1991 02-12-1993 07-04-1994 28-10-1988 26-02-1989 02-11-1988 19-06-1991 16-07-1996 30-12-1992 26-11-1993 27-07-1994 30-11-1994 17-01-1989 19-01-1998 28-03-1989 05-10-1996 04-01-1997 25-06-1991 28-04-1992 21-01-1994 12-10-1993

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/11251

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0305097 A		US 5422115 A ZA 8806322 A	06-06-1995 30-05-1989
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WO 0004169 A	27-01-2000	AU 5505199 A EP 1100933 A	07-02-2000 23-05-2001